DNA examination of ancient dental pulp incriminates typhoid fever as a probable cause of the Plague of Athens

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Summary
Background: Until now, in the absence of direct microbiological evidence, the cause of the Plague of Athens has remained a matter of debate among scientists who have relied exclusively on Thucydides' narrations to introduce several possible diagnoses. A mass burial pit, unearthed in the Kerameikos ancient cemetery of Athens and dated back to the time of the plague outbreak (around 430 BC), has provided the required skeletal material for the investigation of ancient microbial DNA.
Objective: To determine the probable cause of the Plague of Athens.
Method: Dental pulp was our material of choice, since it has been proved to be an ideal DNA source of ancient septicemic microorganisms through its good vascularization, durability and natural sterility.
Results: Six DNA amplifications targeted at genomic parts of the agents of plague (*Yersinia pestis*), typhus (*Rickettsia prowazekii*), anthrax (*Bacillus anthracis*), tuberculosis (*Mycobacterium tuberculosis*), cowpox (cowpox virus) and cat-scratch disease (*Bartonella henselae*) failed to yield any product in 'suicide' reactions of DNA samples isolated from three ancient teeth. On the seventh such attempt, DNA sequences of *Salmonella enterica* serovar Typhi were identified providing clear evidence for the presence of that microorganism in the dental pulp of teeth recovered from the Kerameikos mass grave.

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Conclusion: The results of this study clearly implicate typhoid fever as a probable cause of the Plague of Athens.

Introduction

Until today, all data pertaining to the cause of the plague that devastated Athens in 430—426 BC are based on the account of the epidemic, as reported by the fifth century BC Greek historian Thucydides,[1] who himself was taken ill with the plague but recovered. Despite Thucydides' detailed description,[1,2] researchers have not managed to agree on the identity of the plague.[3] Several pathogens have been putatively implicated in the emergence and spreading of the disease (Table 1), including the Ebola virus,[4—8] the agent of typhoid fever *Salmonella enterica* serovar Typhi, the agent of epidemic typhus *Rickettsia prowazekii*,[9,10] the agent of anthrax *Bacillus anthracis*,[11] the plague bacillus *Yersinia pestis*[12] and even staphylococcal toxic shock syndrome as a complication of influenza.[13,14] Other researchers, based on Thucydides' description of the signs and symptoms of the disease support smallpox,[10,15] Lassa fever, tuberculosis, scarlet fever[2,16] and measles[2,3,16] as being among the most likely causative agents of the Plague of Athens. In any case, the medical puzzle about the actual cause of the Plague of Athens, which brought forward the death of Pericles,[17,18] and precipitated the end of the Golden Age of Athens has remained, until now, unresolved. The profound disagreement on the cause of the plague has been due to the lack of definite microbiological or paleopathological evidence.[3]

In 1994—95, a mass burial site containing at least 150 dead bodies was excavated in the Kerameikos ancient cemetery of Athens (Figure 1). The mass grave constituted a simple pit of rather irregular shape, 6.50 m long and 1.60 m deep. The bodies of the dead were laid in a disorderly fashion, forming more than five successive layers, with no soil between the layers of bodies. At the time of excavation, the bodies closest to the surface lay at a depth of 4.30 m, many layers of soil having accumulated in the intervening centuries. Most bodies were placed in outstretched positions, but many were placed with their heads directed towards the circumference and their feet towards the center of the pit. Dead bodies at the lower level were placed more distant from each other, although the manner of their placement remained more or less as disordered as in the upper layers. Overall, more care for the burial of the dead seemed to have been taken at the lower levels of the mass grave, whereas more carelessness was evident at the upper levels, where the deceased were virtually heaped one upon the other. Among the dead of the upper layer, eight pot burials of infants were found. Contrary to the careless inhumation of the adults that were buried in the same pit, children seemed to have had special care at their burial.[19]

The offerings that were found in the grave comprised a total of 30 small vases scattered among the bodies of the lower layers of the pit. The quality and quantity of the offerings was extremely poor and absolutely disproportionate to such a large number of buried people.[19] Most of the vases that were discovered in the mass grave were dated at around 430 BC, some within the decade of 420 BC and only a few from the last quarter of the fifth century BC.[19]

The hasty and impious manner of burial of the approximately 150 dead, as well as the chronology of the few burial offerings, have linked this site with the outbreak of the Plague of Athens during the first years of the Peloponnesian War, between 430 and 426 BC.[19]

Many archaeologists have wondered at the absence of archaeological evidence related to the victims of the plague. Yet, one should not have expected to find such burial grounds related to the epidemic since, in most cases, the relatives of the deceased undertook other, more common, ways of burial, such as cremations or individual inhumations. Besides, mass graves are rather rare in the ancient Greek world. The few known examples of mass burials in the Classical period have been connected to extreme circumstances, such as the outbreak of lethal epidemic, plague-like diseases. The Kerameikos mass burial evidently did not have a monumental character. In this case, a large number of bodies were thrown one upon the other, rather than buried, in ways of placement that were dictated primarily by the shape and size of the irregular and roughly dug pit. The mass burial of Kerameikos had been completed in such a hasty, improper and impious manner, that any possibility of addressing the dead as victims of war was therefore excluded.[19] Instead, it seemed more likely that the State of Athens hastily buried a large number of hapless and poor dead people as a means to protect its still-surviving population from an epidemic.[19]

The mass burial in the pit of Kerameikos has offered the grounds for a biomedical approach, through the study of the recovered human skeletal remains.[8,20,21] Molecular biology tools have enabled the detection of microbial DNA fragments in ancient skeletal material, thus rendering possible the retrospective diagnoses of ancient diseases.[22—27] In such cases, dental pulp, by virtue of its good vascularization, durability and natural sterility has been shown to be a suitable sample for the recovery of adequate genetic material of microorganisms causing bacteremia.[20,28—31] The recovered sections of ancient microbial DNA may be amplified enzymatically by polymerase chain reaction (PCR) and sequenced to assess the percentage of similarity between parts of the genomes of ancient microorganisms and those of their modern-day counterparts deposited in electronic databases.[30]

Material and methods

The plague-causing pathogen was investigated in ancient dental pulp DNA material of three intact teeth, randomly

Table 1 Theories on the causes of the Plague of Athens in alphabetical order, adapted from Durack et al.[10]

Alimentary toxic aleukia
Anthrax
Bubonic plague
Cholera
Dengue
Ebola/Marburg virus
Ergotism
Erysipelas
Glanders
Influenza
Influenza complicated by toxic shock
    syndrome/Guillain—Barre syndrome
Lassa fever
Malaria
Measles
Meningitis
Rift valley fever
Scarlatina maligna
Scarlet fever
Scurvy
Smallpox
Sweating sickness
Toxic shock syndrome
Tularemia
Typhoid fever
Typhus
Typhus complicated by bubonic plague/dysentery/yellow fever
Yellow fever complicated by scurvy

collected from the Kerameikos mass burial of putative victims of the plague (Figure 1). Two intact teeth collected from a private dental office in Athens served as negative controls against any false-positive amplification of distantly related

Figure 1 Plan of the mass burial in Kerameikos (Reprinted with permission from Baziotopoulou-Valavani E. A Mass Burial from the Cemetery of Kerameikos. In: Stamatopoulou M, Yeroulanou M, editors. Excavating Classical Culture. Recent Archaeological Discoveries in Greece. Studies in Classical Archaeology I. BAR International Series 1031. Oxford, England: Archaeopress; 2002, p. 191).

human genomic sequences, as no other dental archaeological material was available that matched the historical time and location attributes of the material that was investigated in our study. No positive controls were included in the study, thereby excluding all possibility of contamination of the ancient material by DNA from microbes that were to be analyzed. For the same reason all manipulations of ancient teeth, including DNA extraction, were performed in a laboratory located in a different building from the one where the PCR amplifications were performed, and the sequencing procedures were performed in yet another laboratory. None of the tested pathogens or their respective primers had ever been introduced in any of these laboratories, thus minimizing the risk of false-positive results owing to contamination of the ancient material. Furthermore, in order to avoid any bias of the examiner, no data regarding the origin of the teeth or the actual purpose of the test were available to the staff of the laboratories that participated in the study.

All teeth were thoroughly washed and fractured longitudinally. After the opening of the dental pulp cavity was completed, the remnants of the dental pulp (which were powdery in ancient teeth) were scraped off and transferred into sterile tubes. Total dental pulp DNA was isolated using NucleoSpin™ DNA Trace kit (Macherey-Nagel, Germany), following the manufacturer's recommendations. In parallel, extraction of total DNA was performed (using the same kit) in a soil sample washed from ancient teeth.

Two consecutive rounds of PCR amplifications were performed in a reaction volume of 25 μl containing: 5 ng of DNA in 10 mM Tris-HCl (first round) and 2 μl of the first PCR (second round), 20 mM EDTA (pH 8.3), 50 mM KCl, 10 mM Tris-HCl, 1.5 mg MgCl$_2$, 0.1% Triton, 200 μM dNTPs, 0.25 mg of BSA, 2 μM each of the oligonucleotide primers, and 2 units of *Taq* DNA polymerase. Initial denaturation for 2 min at 95 °C was followed by 40 cycles consisting of denaturation for 40 s, annealing for 40 s at a temperature specific for each primer pair, and elongation for 90 s at 72 °C, followed by a prolonged elongation step for 4 min at 72 °C.

The 'suicide' PCR method of DNA amplification[30] that was simultaneously applied in all DNA samples permitted only single use of each pair of primers, which was targeted at microbial DNA sequences not previously investigated in the same laboratory. If a product of the expected size was obtained, its identity was confirmed by DNA sequencing.

The extracted DNA was submitted to successive PCR amplifications using pairs of primers targeting, in turn, the genetic sequences of several pathogens that have been considered as possible causes of the Plague of Athens in the past[2—16] (Table 1), until an expected product was yielded. The order in which the presence of the pathogens was tested was randomly determined.

A total of seven attempts were made, comprising the successive use of primer pairs (shown in Table 2) targeted at genomic sequences of *Y. pestis, R. prowazekii, B. anthracis, M. tuberculosis,* cowpox virus, *B. henselae* and *S. enterica* serovar Typhi. The primers for *Y. pestis, R. prowazekii* and *M. tuberculosis* have been previously used in ancient DNA studies,[25,30] while the primers for *B. anthracis,* cowpox virus and *B. henselae* have been reported to effectively amplify the expected sequences from modest amounts of DNA.[32—34] The primers used for *S. enterica* serovar Typhi were designed

Table 2 Sequence of primers in the order they were used in this study

Organism	Gene(s) and expected length of PCR product	Primers (names and sequence)	Annealing temp. (°C)	Ref.
Y. pestis	*pla* 148 bp	YP12D 5′—CAGCAGGATATCAGGAAACA—3′ YP11R 5′—GCAAGTCCAATATATGGCATAG—3′	52	30
R. prowazekii	*ompB* 190 bp	Rp1 5′—CTCCTCTTACACTTGGTG—3′ Rp2 5′—ACCTGCTTGTAAATTTAAAG—3′	53	30
B. anthracis	*PA* 210 bp	BAPA7 5′—ATCACCAGAGGCAAGACACCC—3′ BAPA6 5′—ACCAATATCAAAGAACGACGC—3′	53	32
M. tuberculosis	*mtp40* 152 bp	MT1 5′—CTGGTCGAATTCGGTGGAGT—3′ MT2 5′—ATGGTCTCCGACACGTTCGAC—3′	66	24
Cowpox virus	*HA* 629—677 bp	CPV1 5′—ATGACACGATTGCCAATACTTC—3′ CPV2 5′—CTTACTGTAGTGTATGAGACAGC—3′	55	33
B. hensellae	*gltA* 380 bp	BQ78 5′—GGGGACACCAGCTCATGGTGG—3′ BQ11 5′—AATGCAAAAAGAACAGTAAACA—3′	51	34
S. enterica serovar Typhi	*osmC* & *clyA* 322 bp	ST2 5′—CGCTTTTCGAGGTTCCTGACG—3′ ST4 5′—CTTGATAAGGTGGAGGCGGGCTTC—3′	58	This study
S. enterica serovar Typhi	*narG* 360 bp	NARG1 5′—GTTCACTTCCTGCCATGAGGAGCG—3′ NARG2 5′—TCAACCCATGGGGTGAACTG—3′	58	This study
H. sapiens	*FV* 175 bp	FVM1 5′—GCAGATCCCTGGACAGTC—3′ FVM2 5′—TGTTATCACACTGGTGCTAA—3′	55	36

according to previously reported sequences in successful PCR amplifications of the region under study.[35]

A second PCR for *S. enterica* serovar Typhi was performed using newly designed primers (Table 2) targeted at the *narG* gene of *S. enterica* serovar Typhi. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining. DNA fragments of interest were extracted from agarose gel using a gel extraction column kit (Qiagen), according to the manufacturer's recommendations. Both strands of the obtained fragments were directly sequenced by cycle sequencing and dye terminator methodologies, using the AmpliTaq cycle sequencing kit (Perkin—Elmer) and an automatic ABI Prism 377 DNA Sequencer (Applied Biosystems). Subsequently, part of the gel-extracted PCR products were cloned in a TOPO TA™ 2.1 plasmid vector (Invitrogen) and sequenced using the M13 primers recommended by the manufacturer. All positive PCR amplifications of aDNA were repeated independently three times in two different laboratories by two different operators.

In order to assess the preservation of DNA in ancient dental pulp, PCR amplification of a human genomic sequence in extracted total DNA from teeth and soil wash was attempted. A region of coagulation factor V gene was amplified as previously described[36] (Table 2) and analyzed by agarose gel electrophoresis.

Results

The presence of preserved DNA sequences in ancient dental pulp was verified by successful amplification of the 175 bp fragment of the human factor V gene in ancient and modern teeth, but not in the sample extracted from the soil wash.

The first six DNA amplifications, which were targeted at genomic parts of the agents of the plague (*Yersinia pestis*), typhus (*Rickettsia prowazekii*), anthrax (*Bacillus anthracis*), tuberculosis (*Mycobacterium tuberculosis*), cowpox (cowpox virus) and cat-scratch disease (*Bartonella henselae*) failed to yield any product in 'suicide' reactions of ancient DNA samples and modern controls. On the seventh such attempt, following the application of primers ST2 and ST4, two products were detected by agarose gel electrophoresis in all three ancient teeth samples: one of the expected size of 322 bp and another one of a size of 440 bp. The two fragments were extracted from gel, and DNA sequencing revealed that the first PCR product had a highly homologous sequence (96%) to parts of genes *osmC* (encoding osmotically inducible protein C) and *clyA* (encoding cytolysin A) of *S. enterica* serovar Typhi. The intervening sequence displayed lower sequence homology (80%). The second product shared limited homology with any known sequence deposited in GenBank.

No product was yielded following the application of the same primers under the same laboratory conditions on the negative controls and also, on the soil sample washed off the ancient teeth.

In order to further investigate the putative presence of *S. enterica* serovar Typhi in the examined ancient DNA, a second
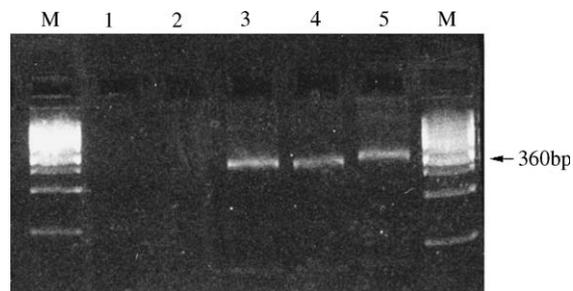

Figure 2 Agarose gel electrophoresis of PCR products using primers corresponding to *narG* gene of *S. enterica* serovar Typhi. M: size marker; 1,2: DNA samples from modern teeth; 3—5: DNA samples from ancient teeth.
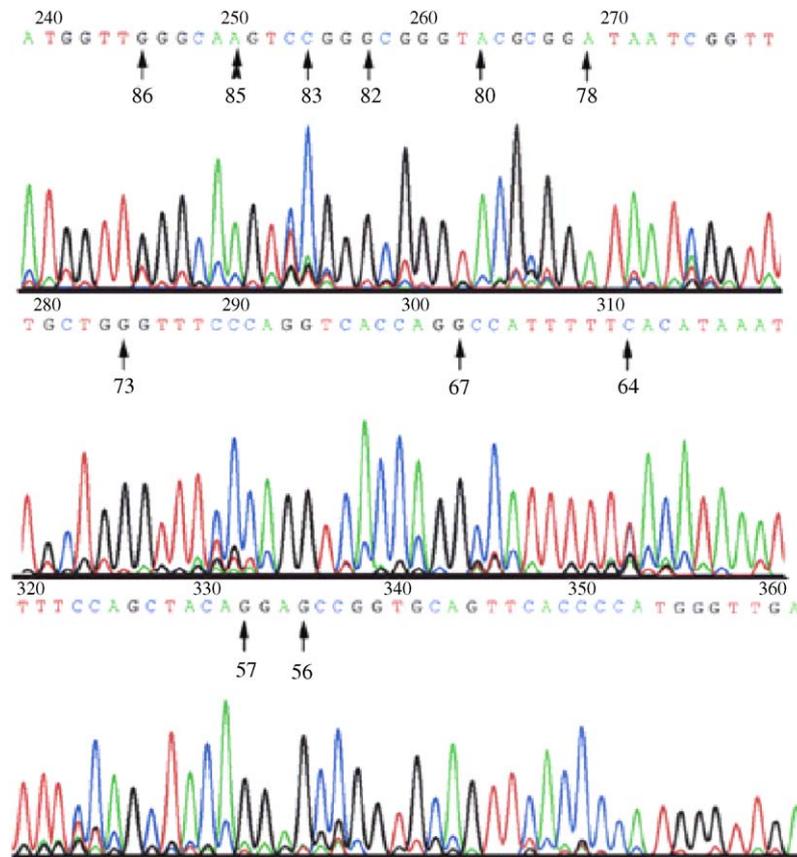
Figure 3 DNA sequence of a region of the *narG* gene corresponding to the modern sequence of *S. enterica* serovar Typhi. Base alterations from the modern sequence are indicated by arrows and codon numbers (see also Table 4). Most of them are silent polymorphisms, since they do not alter the encoded amino acid. A mutation resulting in an amino acid change [**ATG85TTG (Met → Leu)**] is shown with a bold arrow.

pair of primers was used for PCR amplification of another gene, *narG*, which encodes for the alpha chain of the nitrate reductase 1, an important enzyme of anaerobic respiration.[37,38] The yielded PCR product of 360 bp shared 93% sequence homology with *S. enterica* serovar Typhi[37,38] (Figures 2 and 3, Tables 3 and 4). The closest known relative (of *S. enterica*), *Salmonella typhimurium*[39] showed less than 91% homology relevant to two genes (*osmC* and *narG*) and even lacks the entire *clyA* gene.[35] Other bacteria showing more than 80% and less than 88% homology to the identified sequences from the recovered ancient DNA samples were *Escherichia coli* (Table 3), *Erwinia carotovora* and *Shigella flexneri*.

Further analysis of the 240 bp of the *narG* sequence, which was clearly observed in both strands after direct sequencing and cloned PCR-sequencing, revealed 28 nucleotide alterations from the present day Ty2 strain of the typhoid fever agent (Table 4, Figure 3). These include 25 single-base polymorphisms in the third position of a codon that do not alter its genetic meaning, practically excluding the possibilities of either accidental chemical damage of particular nucleotides or amplification of a chimeric PCR product. In addition, three mutations resulting in amino acid changes were detected (Met85Leu, Met118Ile and Leu120Met), but their effect in the spatial conformation and the activity of the *narG* gene product, which is involved in anaerobic respiration, is

unknown. In this study, the sequence of only a 240 bp fragment was analyzed, because that constituted the main part of the product that was clearly read with no background in both strands of all samples and also, because the purpose of our research was simply to identify the pathogen that caused the Plague of Athens and not to sequence its complete genome.

Discussion

In this study, we investigated the plague-causing pathogen by performing 'suicide' PCR amplification in ancient dental pulp DNA material collected from three teeth of putative victims of the Plague of Athens. Their bodies were buried in a mass grave that was excavated in the outskirts of the Kerameikos ancient cemetery of Athens and was dated, through archaeological site documentation, to the exact era of the Plague of Athens.[19]

Teeth were our material of choice, because DNA remnants from systemic pathogens causing bacteremia have been shown by Drancourt et al.[28] and Raoult et al.[30] to be present in ancient dental pulp, which is considered well protected from any natural contamination.[40] It should be mentioned that the suitability of dental pulp for ancient DNA analysis has been questioned by Gilbert et al.[41] who suggested that

Table 3 Comparison of the obtained sequence of the *narG* gene region after cloning (aDNA) with modern sequences of *S. enterica*, *S. typhimurium* and *E. coli* (the differences are shown in bold)

aDNA	GTTCACTTCCTGCCATGAGGAG
S. enterica (AE016839.1)	GTTCACTTCCTGCCATGAGGAG
S. typhimurium (AE008778.1)	GTTCACTTCCTGCCATGAGGAG
E. coli (AE016760.1)	GTTCACTTCCTGCCA**GG**AGGAG
aDNA	CGAACAAA ACC ACCACGACCACGCGCCTGTTT GAAGCTTTTGGCTTTATC
S. enterica	CGAACAAA**GCCGC**CACGACCACGCGCCTGTTT GAAGCTTTTGGCTTTATC
S. typhimurium	CGAACAA**T GCCGC**CACGACCACGCGCCTGTTT GAAGCTTTTGGCTTTATC
E. coli	CGAACAAAACCACC**GCGT**CCACGCGCCTG**C**TT **A**AAGCTTTT**C**GCTTTATC
aDNA	GGCATCTTCAATGATGGAGGCCCATGCCTCTACCGGATCGCGGTGCAGTTT
S. enterica	GGCATCTTCAATGATGGAGGCCCATGCCTCTACCGGATCGCG**A**TGCAGTTT
S. typhimurium	GGCATCTTCAATGAT**C**GAGGCCCATGCCTCTACCGGATCGCG**A**TGCAGTTT
E. coli	GGC**G**TCTTCAATGAT**AGA**AGCCCATGCCT**C**A**A**CCGGATCGC**T**ATGCAGT**CG**
aDNA	CTTGGCCTCGCGCCACAGTTTGATCAGGCGTTTGCGCATCAGCGGGTATT
S. enterica	**TTT**CGCCTCGCGCCACAT**C**TT**C**AT CAGA**C**GTTTGCG CAT**T**AGCGG**A**TATT
S. typhimurium	**TTT**CGCCTCGCGCCACAT**C**TT**C**AT CAGA**C**GTTTGCG CAT**T**AGCGG**A**TATT
E. coli	CTT**C**GC**T**T**C**ACGCCACAT**TTT**CAT CAGGCGTTTGCGCATCA**T**CGGGTATT
aDNA	TCAGGCGGTTGGCGCTGTACAGATACCAGGAGTAACTGGCGCCACGTGGG
S. enterica	TCAG**T**CGGTT**A**GCGCTGTA**G**AGATACCAGGAGTAAC TT**G**C**CCC**G**CGC**GGG
S. typhimurium	TCAG**T**CGGTT**A**GCGCTGTA**G**AGATACCAGGAGTA**G**CTT**G**C**CCC**G**CGC**GGG
E. coli	TCAGGCGGTT**A**GCGCTGTA**T**AGATACCAGGAGTA**G**CTGGC**ACC**G**CGC**GGG
aDNA	CAACCGAGCGGTTCATGGTTGGGCAAGTCCGGGCGGGTACGCGGATAATC
S. enterica	CA**G**CCGAGA**G**GTTCATGGTT**T**GGCATGTC**A**GGA**C**GGGT**G**CGCGGG**T**AATC
S. typhimurium	CA**G**CCG**C**GAGGTTCATGGTT**T**GGCATGTC**A**GGA**C**GGGT**G**CGCGGG**T**AATC
E. coli	CA**G**CCG**C**GAGGTTCATGGTT**T**GGCA**GG**TCCGGGC**G**AGTACGCGGATA**GTC**
aDNA	GGTTTGCTGGGTTTCCCAGGTCACCAGGCCATTTTTCACATAAATTTTCC
S. enterica	GGTTTGCTG**T**GTTTCCCAGGTCAC CAGA**C**CATTTTT**G**ACATAAATTTTCC
S. typhimurium	GGTTTGCTG**T**G**T**GTTTCCCAGGTCACCAG**A**CCATTTT **G**ACATAAATTTTCC
E. coli	**AG**T**CTGCTGGGTTTCCCAGGT**G**ACCAG**A**CC**G**TTTTT**G**AC**G**TA**G**ATTTTCC
aDNA	AGCTACAGGAGCCGGTGCAGTTCACCCCATGGGTTGA
S. enterica	AGCTACA**AGAAC** CGGTGCAGTTCACCCCATGGGTTGA
S. typhimurium	AGCTACA**AGAAC** CGGTGCAGTTCACCCCATGGGTTGA
E. coli	AGCT**G**CAGGAGCCGGTGCAGTT**T**ACCCC**G**TGGGT**A**GA

The GenBank accession numbers are given in parentheses. Note that the last two thirds of the sequence was clearly read after both direct and cloning-based sequencing.

environmental DNA contamination might persist within external layers of teeth despite external decontamination. In their experience, these contaminants entered the DNA extraction after longitudinal fracturing of teeth and scraping of pulp, possibly via contact with gloves when the teeth were manipulated, despite high levels of preventive care.[41] They proposed a method of encasing teeth in silicone and removal of dental pulp residue and dentine by drilling into the root tip, thus achieving reduction of contamination levels.[41] In response, Drancourt and Raoult[42] defended their research methodology arguing that dental pulp is a well-vascularized soft tissue in contrast to dentine or bone and is sterile in patients without bacteremia.[30,40] Viable microorganisms as well as their nucleic acids have been detected in dental pulp of infected patients and experimentally infected animals,[29,43] while dentine has never been tested experimentally for this purpose. Complete knowledge of dental anatomy allows for considering dental pulp to be more suitable than dentine for ancient DNA analysis, provided that extreme preventive measures for avoiding contamination are taken.

Various PCR methods have been used for the identification of pathogenic microbes in ancient human samples,[23−28,30,41] usually applying highly preventive measures in order to minimize the risk of false-positive results, due to sample contamination by previously attempted analyses or natural flora. In this study absolute preventive measures were taken to avoid contamination of the ancient material by the virulent agents that were to be examined. These measures included the absence of the pathogens themselves or a previously attempted extraction or PCR amplification of the target DNA in the laboratories that were used in the study. The 'suicide' PCR amplification procedure that was followed, further ensured the absence of amplicon contamination in the laboratory of the ancient material, since PCR primers were designed to hybridize to targets outside genomic regions previously targeted in the same laboratory.[30] This methodology requires that positive controls should never be

Table 4 Detected *narG* gene differences between the ancient and modern strain of *S. enterica* serovar Typhi

Codon	Modern	Ancient
Mutations		
85	ATG (Met)	TTG (Leu)
118	ATG (Met)	ATC (Ile)
120	ATG (Met)	CTG (Leu)
Polymorphisms		
56	GGT (Gly)	GGC (Gly)
57	TCT (Ser)	TCC (Ser)
64	GTC (Val)	GTG (Val)
67	GGT (Gly)	GGC (Gly)
73	ACA (Thr)	ACC (Thr)
78	TAC (Tyr)	TAT (Tyr)
80	CGC (Arg)	CGT (Arg)
82	CGT (Arg)	CGC (Arg)
83	CCT (Pro)	CCG (Pro)
86	CCA (Pro)	CCC (Pro)
89	CCT (Pro)	CCG (Pro)
91	GGC (Gly)	GGT (Gly)
93	CCG (Pro)	CCA (Pro)
94	CGC (Arg)	CGT (Arg)
95	GGG (Gly)	GGC (Gly)
96	GCA (Ala)	GCC (Ala)
102	CTC (Leu)	CTG (Leu)
105	GCT (Ala)	GCC (Ala)
107	CGA (Arg)	CGC (Arg)
110	TAT (Tyr)	TAC (Tyr)
112	CTA (Leu)	CTG (Leu)
116	CGT (Arg)	CGC (Arg)
119	AAG (Lys)	AAA (Lys)
124	GCG (Ala)	GCC (Ala)
125	AAA (Lys)	AAG (Lys)

used in such studies of ancient DNA,[30] an approach that was also followed in our study.

Cooper and Poinar[44] have proposed nine criteria for validating ancient DNA studies including: (a) physically isolated work area, (b) control amplifications, (c) appropriate molecular behavior of PCR products, (d) reproducibility of results, (e) cloning of amplified products, (f) survival of associated human DNA remains, (g) independent replication by sequencing in independent laboratories, (h) biochemical preservation studies of DNA, and (i) quantitation of copy number of target DNA using competitive PCR. In this study, the first seven criteria were met, while the remaining two will follow in subsequent studies. As part of the negative controls of contamination suggested by Cooper and Poinar, some researchers have used extraction and PCR blanks (i.e., water instead of sample),[41] but in this study we preferred to use soil wash as a negative extraction control and DNA from modern teeth as negative PCR controls.

Through the study and the analysis of Thucydides' descriptions, several infectious diseases have been proposed as candidate causative agents of the plague of Athens[3—16] (Table 1). In our research we randomly and successively investigated the putative presence of as many of them as possible, until a positive result was observed.

Six putative causative agents of the Plague of Athens were separately tested including, in turn, the agents of plague (*Yersinia pestis*), typhus (*Rickettsia prowazekii*), anthrax (*Bacillus anthracis*), tuberculosis (*Mycobacterium tuberculosis*), cowpox (cowpox virus) and cat-scratch disease (*Bartonella henselae*), all of which yielded no product. These negative results might be attributed to either the non-existence of the respective investigated pathogens or the non-preservation of a tested DNA sequence over time. Testing for multiple loci of the same microorganism might address the issue of variable preservation of its genome, but in this study we decided to use the 'suicide' PCR approach and concentrate our in-depth analysis on a possible positive finding. Indeed, the seventh such attempt, targeted at typhoid fever agent (*Salmonella enterica* serovar Typhi) eventually yielded a product of expected size.

The PCR and DNA sequencing results of this study demonstrate that an ancient strain of *Salmonella enterica* serovar Typhi was present in the dental pulp of three randomly selected individuals that were buried in a mass grave, dated back to the era of the Plague of Athens. The fact that the preservation of studied microbial aDNA sequences was good enough for molecular detection and analysis in all three teeth might reflect the possible large amount of *S. enterica* serovar Typhi cells due to bacteremia. Dental pulp is known to be appropriate for the detection of bacteremic pathogens[29,43] and typhoid fever is indeed a deadly septicemic pathogen.[45,46] Therefore, in view of the results of our study, we conclude that a strain of *Salmonella enterica* serovar Typhi, or a bacterial species very closely related to it, if not *S. enterica* serovar Typhi-*stricto sensu,* was involved in the epidemic that devastated Athens in 430—426 BC. The 93% homology of the ancient DNA sequence to the respective genome of the present day strain of *Salmonella enterica* serovar Typhi is regarded as high enough to allow the above conclusion to be considered as safe. If another, yet unknown pathogen (and not an ancestral strain of *S. enterica*) 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* and *E. coli*. The identified genomic differences between the recovered DNA from Kerameikos teeth and the present day strains of *S. enterica*[37,38] (most of which do not alter the codon meaning (cited in Table 4)) provide further clear evidence that the recovered and investigated microbial DNA sample is an ancestral strain of *S. enterica*.

The fact that *Salmonella enterica* serovar Typhi sequences were independently amplified in three different individuals reinforces the results of this study. Furthermore, the fact that six alternative virulent agents were previously investigated as candidate causes of the plague but were not identified further reinforces the above assumption. Any possibility that the presented products could have resulted from a soil microorganism was excluded, since the application of the same primers under the same conditions to the soil washed from the ancient teeth, failed to yield any product.

For an infectious disease to be considered as a likely cause of the Plague of Athens, it must, above all, have existed at that time.[3] Infectious diarrheas and dysentery, as described by the ancients, imply that typhoid fever was an endemic problem in the ancient world.[45] Because of the absence of

direct microbiological evidence, until now, the diagnosis of the cause of the Plague of Athens was based exclusively on Thucydides' narrations, taking their validity and reliability for granted. In addition, it is generally assumed that no key clinical features were omitted and the description of the disease was as accurate as possible at the time, even though some parts of Thucydides' great history were written in retrospect, as much as 20 years after the recorded facts.[10] Although a keen observer, a careful recorder of events and a victim of the disease himself, Thucydides may not have been able to weigh the relative significance of the variable clinical manifestations of the plague. The writer may have stressed trivial signs and symptoms at the expense of important ones.[10] Even though the description of the plague reflects Thucydides' familiarity with medical terminology, it is important to remember that the writer was not a physician but an historian.

Our molecular diagnosis of typhoid fever is consistent with some of the key clinical features reported by Thucydides,[1–3] including the fever, the rash and the diarrhea.[3] Other features of the disease, as cited in Thucydides' work, such as the acuteness of its onset,[3,10] are inconsistent with the typical present-day form of typhoid fever.[46] This inconsistency may be explained by a possible evolution of typhoid fever over time, which means that the disease may not manifest itself in the same fashion today[46] as in the past.[18] The virulence of infectious diseases varies among populations and over time as the populations are repeatedly subjected to successive attacks of the disease.[16] Genomic differences between the ancient and present day *S. enterica* serovar Typhi strains, like the ones identified in our study, may offer some reasonable explanation for the differences in the clinical features between the Plague of Athens[1,2] and the present day form of typhoid fever.[46] Alternatively, the concurrent presence of a plurality of infectious diseases in besieged Athens of 430–426 BC cannot be excluded,[10] allowing for the variable clinical manifestations of Thucydides' report of the plague.[3] It would have been extremely difficult if not impossible, for Thucydides or any other observer, to distinguish between two or more such diseases at that time.[10]

In conclusion, the results of this study incriminate typhoid fever as a probable cause of the Plague of Athens. Considering the overcrowding and insanitary conditions (especially regarding the water supplies) within the walls of the besieged Athens, a typhoid epidemic would have been likely to break out either as the solitary cause of the plague or as an minor epidemic adjunct to a yet unidentified agent of the major one. However, additional investigation of DNA material from the Kerameikos mass grave is needed to further test the results of this study and address related factors such as ancient DNA preservation.

Acknowledgements

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