Chapter 10
Typhoid Fever Epidemic in Ancient Athens

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Abstract Molecular evidence, resulting from investigation and analysis of ancient DNA, has identified the presence of *Salmonella enterica* serovar Typhi in victims of the Plague of Athens, thereby incriminating typhoid fever as a likely cause of the epidemic. Current clinical and epidemiological scientific data, related to modern-day typhoid, correlate well to the signs and symptoms of the disease as Thucydides has described them, whereas their apparent differences may be reasonably explained. The most striking hypothesis is that the ancient *S. typhi* strain may constitute the ancestral original strain of the pathogen, capable of affecting both human and animal hosts. The genomic evolution of the ancient *Salmonella typhi* strain over time may provide a satisfactory explanation for the diminished morbidity and the varying clinical symptomatology of modern-day typhoid fever. Further investigations, implementing DNA sequencing techniques of the ancient strain of *S. enterica*, may elucidate its genetically determined differences from its modern counterpart, thus facilitating new approaches to preventing or treating typhoid fever epidemics.

10.1 The Plague of Athens – Historical Background

At many historical crossroads, major epidemics have been shown to have influenced the rise and fall of several great civilisations. Such an epidemic is, undoubtedly, the one that literally decimated the population of the city-state of Athens around 430–426 B.C. – generally known as the Plague of Athens [Thucydides §2.47–2.54 (note that all references to Thucydides' description are taken from C.F. Smith's 1919 translation (Thucydides 1919)]. This epidemic was a decisive factor that changed the balance of power, thereby determining the outcome of the Peloponnesian War of Athens against Sparta, thus ending the Golden Age of Pericles (Thucydides §2.47–2.54; Longrigg 1980; Soupios 2004). As a direct

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consequence, Athenian predominance in the Mediterranean Sea declined, thereby greatly affecting the route of history.

The Plague broke out during the siege of Athens by the Spartan army early in the summer of 430 B.C. (Thucydides §2.47–2.54). After a brief remission in 428 B.C., the epidemic reemerged in the winter of the following year and lasted until the winter of 426 B.C. About one-third of the Athenian population, one-fourth of their army and their charismatic leader Pericles, perished in the epidemic.

Until recently, all data pertaining to the Plague and its clinical characteristics were based on the account of the epidemic as reported by the fifth century B.C. Athenian historian Thucydides, who himself was taken ill with the Plague but recovered. In his famous history of the Peloponnesian War, Thucydides gave a detailed description of the epidemic (Thucydides §2.47–2.54) that has since formed the basis of several hypotheses regarding its cause. Thucydides' records describe major signs and symptoms of the disease as well as other associated events and behaviour of affected and non-affected Athenians. The validity and reliability of these narrations is taken for granted.

10.2 Hypotheses on the Cause of the Plague of Athens

Although extensively informative, the narration of Thucydides regarding the Plague of Athens does not correspond exactly to the characteristics of any single disease as it is known today. This fact has prompted various authors to suggest about 30 possible pathogens that might putatively be implicated in the emergence and spread of the disease, based on Thucydides' description of its signs and symptoms. These included the causative agents of smallpox, Lassa fever, measles, scarlet fever, tuberculosis (*Mycobacterium tuberculosis*), epidemic typhus (*Rickettsia prowazekii*), anthrax (*Bacillus anthracis*), typhoid fever (*Salmonella enterica* serovar Typhi), plague (*Yersinia pestis*), Ebola, and even staphylococcal toxic shock syndrome as a complication of influenza (Shrewsbury 1950; Page 1953; Littman and Littman 1969; Langmuir et al. 1985; Holladay 1986; Scarrow 1988; McSherry and Kilpatrick 1992; Olsen et al. 1996, 1998; Holden 1996; Perry and Fetherston 1997; Durack et al. 2000; Cunha 2004). Nevertheless, each one of the proposed "most likely" causative agents of the Plague of Athens explained only a percentage of the epidemic characteristics described by Thucydides.

In any case, the mystery of the Plague that precipitated the end of the Golden Age of Athens has, until recently, remained unresolved due to the lack of definitive archeological and biological evidence.

10.3 A Mass Burial Site of Plague Victims

The skeletal material necessary for an objective investigation of the cause of the Plague of Athens was provided by a recent excavation (1994–1995) conducted in Kerameikos' ancient cemetery of Athens.

In this excavation, a mass burial site was discovered in the outskirts of Kerameikos (Baziotopoulou-Valavani 2002). The mass grave was a simple 6.5 m-long pit of rather irregular shape that contained the remains of at least 150 individuals of various ages (men, women and children). The dead bodies were laid in a disorderly manner, most of them in outstretched positions, but several were placed with their feet directed towards the pit centre and their heads towards the circumference (Baziotopoulou-Valavani 2002). They formed more than five successive layers, without any intervening soil between them. At the lower level, the deceased were more distant from each other, although their manner of placement was as disordered as in the upper layers. It seemed that more care during burial had been taken at the lower levels of the mass grave, while at the upper levels the dead were virtually heaped one upon the other (Baziotopoulou-Valavani 2002). In the upper layer, eight pot burials of infants were found, indicating that special care was taken for them in contrast to the careless piling of adults in the same pit (Baziotopoulou-Valavani 2002).

A few offerings of about 30 small vases were found scattered among the bodies of the lower layers of the grave (Baziotopoulou-Valavani 2002). The quality and quantity of these offerings were extremely poor and absolutely disproportionate to such a large number of buried people. Most of the discovered vases were dated at around 430 B.C., using accepted chronological techniques (Baziotopoulou-Valavani 2002). The chronology of the few burial offerings as well as the hasty and impious manner of burying about 150 dead were associated with the outbreak of the Plague of Athens during the first years of the Peloponnesian War, between 430–426 B.C. (Baziotopoulou-Valavani 2002).

The absence of archaeological evidence relating to the victims of the Plague is due to the fact that, in most cases, the relatives of the deceased usually undertook other ways of burials, such as cremations or individual inhumations (Thucydides §2.47–2.54).

The mass burial of Kerameikos evidently did not have a monumental character. It had been completed in such a hasty, improper and uncommonly impious manner, that any possibility of addressing the dead as victims of war was therefore excluded (Baziotopoulou-Valavani 2002). Instead, the most likely explanation is that the authorities of the City of Athens hastily buried a large number of poor and hapless dead Plague victims as a means of protecting its still surviving population from the epidemic (Baziotopoulou-Valavani 2002). Mass graves are rather rare in the ancient Greek world, and the few known such examples in the Classical period have been connected to extreme circumstances such as the outbreak of lethal, epidemic plague-like diseases.

In this case, a large number of dead bodies were thrown one upon the other (rather than buried) in ways that were dictated primarily by the shape and size of the irregular and roughly dug pit.

Therefore, the mass burial pit of Kerameikos offered the opportunity for a biomedical evidence-based approach towards resolving the mystery of the agent that caused the Plague of Athens, through the study of the recovered human skeletal remains.

10.4 DNA Extraction From Teeth of Plague Victims

The molecular detection of microbial DNA sequences in ancient skeletal material has made possible the retrospective diagnosis of ancient infectious diseases (Pääbo 1989; Taylor et al. 1996, 1999; Taubenberger et al. 1997; Nerlich et al. 1997; Kolman et al. 2000; Raoult et al. 2000, 2006). Recovered DNA fragments of ancient microorganisms may be enzymatically amplified by various methods of polymerase chain reaction (PCR) and consequently sequenced to assess their similarity with their modern-day counterparts deposited in electronic databases (Taylor et al. 1996, 1999; Nerlich et al. 1997; Taubenberger et al. 1997; Kolman et al. 2000; Raoult et al. 2000, 2006; Cunha 2004). In addition to highly accurate molecular methods, extreme preventive measures must be applied in order to minimise the risk of false-positive results due to sample contamination by previously attempted analyses or naturally occurring microorganisms.

In the case of the Plague of Athens, the material of choice was intact teeth (as observed macroscopically and verified by X-radiographs) randomly collected from three different Plague victims of the Kerameikos mass grave, because DNA remnants from systemic pathogens causing bacteraemia had previously been shown to be present in ancient dental pulp (Holden 1998; Raoult et al. 2000, 2006). By virtue of its good vascularisation, durability and natural sterility, dental pulp is considered well protected from any external contamination. Only in case of bacteraemia, would the ancient dental pulp have trapped genetic material of the pathogenic microorganism in amounts adequate for study (Holden 1998; Aboudharam et al. 2000; Raoult et al. 2000, 2006; Drancourt and Raoult 2002).

Since no other dental archaeological material was available, matching the historical time and location attributes of the material under study, two modern intact teeth served as negative controls against any false-positive amplification of distantly related human genomic sequences (Papagrigorakis et al. 2006a). In addition, a soil sample washed from ancient teeth served as a negative control of external contamination during DNA extraction (Papagrigorakis et al. 2006a). No positive controls were included, thereby excluding any possible contamination of the ancient material by DNA from the microbes under study. For the same reason, the steps of DNA extraction, PCR amplification and DNA sequencing reactions were performed in three laboratories located in different buildings (Papagrigorakis et al. 2006a). None of the pathogens under study or their respective primer sequences had ever been introduced into any of these laboratories, thus minimising the risk of false-positive results due 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 analysis were available to the staff of the laboratories that participated in the study (Papagrigorakis et al. 2006a).

Ancient and modern teeth were thoroughly washed and fractured longitudinally. The remnants of the dental pulp, which were powdery in ancient teeth, were scraped off and transferred into sterile tubes. Using a Forensic DNA Trace kit (Nucleospin DNA Trace, Macherey-Nagel, Düren, Germany), total dental pulp

DNA was isolated from all teeth under study as well as from a soil sample washed from ancient teeth (Papagrigorakis et al. 2006a).

10.5 "Suicide" PCR Attempts for Candidate Pathogens

In order to assess the preservation of DNA in ancient dental pulp, PCR amplification of a human genomic sequence in extracted DNA samples was attempted. A region of the coagulation factor V gene was amplified in all samples extracted from ancient and modern teeth, but not in the sample extracted from the soil wash (Papagrigorakis et al. 2006a).

The investigation of the Plague-causing pathogen was effected by two consecutive rounds of "suicide" PCR amplification performed simultaneously in all DNA samples corresponding to all studied teeth and the soil wash (Papagrigorakis et al. 2006a). The previously described "suicide PCR" method (Raoult et al. 2000) permitted only a single use of each primer pair, targetted successively at candidate microbial DNA sequences, until a product of the expected size was obtained and visualised by ethidium bromide staining after agarose gel electrophoretic analysis. The identity of this product then had to be confirmed by DNA sequencing.

The presence of seven putative causative agents of the Plague of Athens was randomly and successively investigated. No PCR product was yielded in "suicide" reactions of ancient DNA samples and controls using primers for sequences of the agents of plague (*Yersinia pestis*), typhus (*Rickettsia prowazekii*), anthrax (*Bacillus anthracis*), tuberculosis (*Mycobacterium tuberculosis*), cowpox (*Cowpox virus*) and cat-scratch disease (*Bartonella hensellae*) (Papagrigorakis et al. 2006a).

The seventh such attempt, targetted at a sequence of the agent of typhoid fever (*Salmonella enterica* serovar Typhi), eventually yielded, in all three ancient teeth, a product of the expected size, corresponding to a 322 bp fragment containing parts of the *osmC* (encoding osmotically inducible protein C) and *clyA* (encoding cytolysin A) genes (Papagrigorakis et al. 2006a). DNA sequencing confirmed that the sequence of the PCR product was highly homologous (96%) to parts of both genes of *S. enterica* Typhi, while the intervening sequence displayed lower sequence homology (80%) (Papagrigorakis et al. 2006a). On the contrary, no product was obtained using the same primers under the same laboratory conditions on the negative controls (modern teeth and soil wash) (Papagrigorakis et al. 2006a). In order to confirm the putative presence of *S. enterica* Typhi genomic sequences in the examined ancient dental pulp samples, another "suicide" PCR was attempted using a pair of primers targeted at the *narG* gene (encoding the alpha chain of nitrate reductase 1). (Parkhill et al. 2001; Deng et al. 2003). The expected PCR product of 360 bp was obtained in all three ancient DNA samples but in none of the three negative controls. DNA sequencing revealed that it shared 93% sequence homology with the respective *narG* gene of *S. enterica* Typhi (Parkhill et al. 2001; Deng et al. 2003; Papagrigorakis et al. 2006a). All positive PCR amplifications of ancient DNA were repeated independently three times in two different laboratories by two different specialists (Papagrigorakis et al. 2006a).

10.6 An Ancestral Strain of *Salmonella enterica* Serovar Typhi?

The identified microbial sequences that existed in the ancient dental pulp of Plague victims were clearly highly homologous to modern sequences of the typhoid fever agent, but they did not match exactly with them. Was this an artifact either due to chemical decomposition of some nucleotides over time or due to amplification of patchy DNA fragments of various origins? Alternatively, did this result suggest that the DNA sequences identified belonged to an ancient strain of *S. enterica* serovar Typhi?

A 240 bp region of the *narG* sequence, which was clearly detected with no background in both strands of all samples after direct sequencing and cloned PCR-sequencing, was further analysed. It contained 28 nucleotide alterations (involving most possible nucleotide swaps) from the present day Ty2 strain of *S. enterica* Typhi (Papagrigorakis et al. 2006a). The great majority of these alterations (25/28) were single-base polymorphisms in the third position of the codon that did not alter its genetic meaning, practically excluding the possibilities of either accidental chemical damage of particular nucleotides or amplification of a chimaeric PCR product (Papagrigorakis et al. 2006a). Only three nucleotide alterations were single-base missense mutations resulting in amino acid changes (Met85Leu, Met118Ile and Leu120Met); the effect of these mutations on the spatial conformation and activity of the *narG* gene product, which is involved in anaerobic respiration, is unclear (Papagrigorakis et al. 2006a).

These data ascertain that an ancient strain of the typhoid fever agent *S. enterica* serovar Typhi was present in the dental pulp of three randomly selected individuals buried in a mass grave of about 150 individuals, which was dated to the era of the Plague of Athens. Both the fact that *S. enterica* Typhi sequences of three genes were independently amplified in three different Plague victims, and the fact that six alternative candidate agents previously investigated as candidate causes of the Plague were not identified, further reinforce the above assumption. Any possibility that the detected PCR products could have resulted from a modern and currently unknown free-living soil bacterium instead of an ancient one was excluded, since, under the same conditions, application of the same primers to soil washed from the ancient teeth failed to yield any product (Papagrigorakis et al. 2006a, 2006b). The fact that the ancient microbial DNA sequences were preserved for more than 24 centuries in good enough condition for molecular detection and analysis in all three studied teeth might possibly reflect the presence of a large amount of *S. enterica* Typhi cells due to bacteraemia. The typhoid fever agent is indeed a deadly septicaemic pathogen, and dental pulp is known to be appropriate for the detection of bacteraemic pathogens (Glick et al. 1991; Raoult et al. 2000, 2006; Aboudharam et al. 2000; Parry et al. 2000).

In conclusion, it seems that a strain of *S. enterica* serovar Typhi (or a bacterial species very closely related to it, if not *S. enterica* Typhi *stricto sensu*) was clearly involved in the epidemic that devastated Athens in 430–426 B.C. (Papagrigorakis et al. 2006a). The 93–96% homology of the ancient DNA sequences to the corresponding sequences of a present day strain of the typhoid fever agent is regarded as high enough to allow the above conclusion to be considered as safe. If another, presently 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* or *E. coli*.

Salmonella typhimurium, which is the closest known relative of *S. enterica* Typhi (McClelland et al. 2001), shared less than 91% homology with the ancient DNA sequences in two genes (*osmC* and *narG)* and even lacks the entire *clyA* gene (Papagrigorakis et al. 2006a; Oscarsson et al. 2002). Other bacteria showing 80–88% homology to the ancient DNA sequences were *Escherichia coli*, *Erwinia carotovora* and *Shigella flexneri* (Papagrigorakis et al. 2006a).

The identified genomic differences (most of which do not alter the codon meaning) between the recovered DNA from teeth of Kerameikos and present day strains of *S. enterica* provide further clear evidence that the recovered microbial DNA probably belongs to an ancestral strain of *S. enterica* (Papagrigorakis et al. 2006a, 2007).

10.7 Thucydides' Narration Revisited

In order to consider an infectious disease as a likely cause of the Plague of Athens, first of all it must have existed at that time (Cunha 2004). Certainly, ancient descriptions of infectious diarrhoeas and dysentery imply that typhoid fever was an endemic problem in the ancient world (Lim and Wallace 2004). Interestingly, Hippokrates, who also lived in the fifth century, described very accurately the symptomatology of typhoid fever, although he used the name "typhus" instead.

Even though some parts of Thucydides' history were written retrospectively (up to a decade after the recorded facts), it is generally assumed that his narrations are reliable and valid. Taking into account the fact that the historian was not a physician, it is assumed that no key clinical features were omitted, thus the description of the epidemic was as accurate as possible at the time (Durack et al. 2000). Also Thucydides was a keen observer and a careful recorder of events, as well as himself being a victim of the disease, he may not have been able to weigh the relative significance of the variable clinical manifestations of the Plague. Although the historian may have stressed trivial signs and symptoms at the expense of important ones (Durack et al. 2000), the molecular diagnosis of typhoid fever is consistent with some of the key characteristics and clinical features reported by Thucydides (Thucydides §2.47–2.54; Page 1953; Cunha 2004):

1. The sudden outbreak of the disease in the city of Athens has been linked either to incoming travellers (carriers of the disease) from other countries like Egypt to the port of Piraeus, or to the speculated poisoning of the water reservoirs by the Spartans (Thucydides §2.47–2.54). Both etiologies apply to what is observed

even today regarding ways of contracting and spreading typhoid fever in visitors and residents in areas of developing countries endemic for typhoid fever (Hoffner et al. 2000; Parry et al. 2002; Connor and Schwartz 2005; Bhan et al. 2005).

2. The crowded and unsanitary conditions in besieged Athens of 430 B.C. (Thucydides §2.47–2.54) undoubtedly must have favoured the spread of the epidemic, as is usually the case in modern-day typhoid epidemics in developing countries (Parry et al. 2002; Connor and Schwartz 2005; Bhan et al. 2005), especially where the water supplies are contaminated by the pathogen or poor housing does not facilitate personal hygiene (Black et al. 1985; Luby et al. 1998; Mermin et al. 1999; Gasem et al. 2001). These conditions were progressively made even worse for the besieged ancient Athenians by the continuing influx of refugees from the countryside into the city, where they lived in crowded conditions and in close contact with the sick (Thucydides §2.47–2.54).
3. The contagious nature of the disease is emphasised by the fact that relatives, friends and doctors contracted the disease by coming into close contact with affected people (Thucydides §2.47–2.54). Social or physical contact with patients with typhoid fever has also been identified as a risk factor for contracting the disease today (Luxemburger et al. 2001).
4. Thucydides also mentions that all other cases of sickness in the ancient Athens of 430 B.C. ended in the Plague (Thucydides §2.47–2.54). In modern times also, contracting typhoid is more likely in patients with weaker constitutions, e.g. immunosuppressed patients (Hoffner et al. 2000; Bhan et al. 2002).
5. No remedy was found even for the best attended Plague-affected Athenians (Thucydides §2.47–2.54). This is not surprising since the only known effective treatment of typhoid fever is the administration of specific antibiotic drugs (Hoffner et al. 2000; Parry et al. 2002; Connor and Schwartz 2005; Bhan et al. 2005), which of course were not available in ancient Athens.
6. Prevention of contracting the disease was not possible; in modern times this is achieved by vaccinating the entire population of typhoid endemic areas (Parry et al. 2002; Connor and Schwartz 2005; Bhan et al. 2005).
7. The major clinical features of the Plague of Athens, as reported by Thucydides, indicate an epidemic of acute onset and rapid progression that ended fatally for most of the affected persons, decimating about one-third of Athenians, including their charismatic leader, Pericles (Thucydides §2.47–2.54). This is also in accord with a diagnosis of typhoid fever, since even though today's worldwide mortality rate from the disease is 1%, it may rise to up to 30–50% in endemic areas, especially if antibiotic treatment is delayed (Hook 1984; Punjabi et al. 1988; Rogerson et al. 1991; Parry et al. 2000).
8. The molecular diagnosis of typhoid fever is consistent with most of the key clinical features reported by Thucydides (Thucydides §2.47–2.54), including the prolonged and violent attacks of headache and fever, the cough and the sore throat, as well as the subsequent abdominal pain, diarrhoea and rash (Hoffner et al. 2000; Cunha 2004). Some other features of Thucydides' report, including confusion, apathetic behaviour (Thucydides §2.47–2.54) may be observed, though much more rarely, in some modern cases (Hoffner et al. 2000).

Other features of the disease, as cited in Thucydides' work (Thucydides §2.47–2.54), are inconsistent with the typical present-day form of typhoid fever (House et al. 2001; Parry et al 2002; Bhan et al. 2005). These include its reported acute onset and its clinical symptoms of conjuctivitis, development of profound ulcers, gangrene of the extremities, and a sensation of intense internal heat (Thucydides §2.47–2.54). These inconsistencies may be attributed to a possible evolution of the typhoid fever pathogen over time, which means that the disease may not manifest itself in the same fashion today, and that it was present in a much more aggressive form in the past (Soupios 2004; Cunha 2004). Alternatively, the concurrent presence of a plurality of infectious diseases in besieged Athens of 430–426 B.C. cannot yet be excluded, allowing for the variable clinical manifestations of Thucydides' report of the Plague (Durack et al. 2000; Cunha 2004). It would have next to impossible for Thucydides or any other observer to distinguish between two or more such diseases at that time (Durack et al. 2000).

The fact that the residents of Athens of 430 B.C. showed no resistance to the advent and the rapid spread of the epidemic (Thucydides §2.47–2.54) indicates that there was no immunisation of the population by any former introduction of the disease, while at the same time the Athenians were probably malnourished due to the siege of their city by the Spartans (Soupios 2004; Cunha 2004). This may also be an explanation of why the severe complications of typhoid fever, such as cardiovascular, pulmonary, respiratory, central nervous system, neuropsychiatric, hepatobiliary, genitourinary, haematologic and other symptoms, that are sometimes observed in neglected or immuno-suppressed cases of modern times (Parry et al. 2002; Huan and DuPont 2005), were typical of the characteristic clinical features of the Plague of Athens (Thucydides §2.47), where no effective treatment was available.

Death from the Athens' epidemic ensued on the 7th–9th day from initiation of symptoms (Thucydides §2.47–2.54), whereas in modern times, in untreated cases the disease ends fatally due to the development of severe complications after 2–3 weeks (Cunha 2004). In addition, in modern typhoid outbreaks, the case fatality rate is higher among the very young and elderly patients (Stuart and Pullen 1946; Parry et al. 2002), whereas in ancient Athens strong and weak constitutions alike were affected (Thucydides §2.47–2.54). This may be attributed to the general immunisation to typhoid epidemics of the population over time, as people were repeatedly subjected to successive attacks of the disease (Shrewsbury 1950). The same conclusion is also reached through the analysis of Thucydides' account of the disease, as those ancient Athenians that recovered were never attacked twice with the same morbidity and mortality (Thucydides §2.47–2.54). Besides, in modern times, although typhoid fever relapses in 5–10% of cases, the second wave usually occurs in milder clinical form, thus verifying the immunising effect of the first attack (Parry et al. 2002; Bhan et al. 2005). Nevertheless, even today typhoid fever is a major health problem on a global scale. Every year there are about 20 million new cases that lead to about 600,000 deaths in the developing world, where overpopulation, inadequate water supplies and hygiene, as well as poor access to health services allow epidemics to spread with tragic results.

Last, but not least, one of the most outstanding features of Thucydides' account of the Plague of Athens' is the reported contraction of the disease by animals (Thucydides §2.47–2.54). This is very interesting since today typhoid fever is strictly adapted to humans (Parry et al. 2002; Bhan et al. 2005). Not excluding the possibility of a combination of diseases occurring simultaneously in ancient Athens (Durack et al. 2000), this finding may fit a working hypothesis of the intriguing possibility that this specific strain of *S. enterica* Typhi, incriminated as the cause of the Plague of Athens, was an ancient strain that was not adapted to human hosts only (Papagrigorakis et al. 2006b), whose existence has been anticipated (Kidgell et al. 2002).

10.8 Future Prospects

Typhoid fever almost certainly played a part in causing the Plague of Athens, either exclusively or in combination with another (and so far unknown) infection. Genomic differences between ancient and present day *S. enterica* serovar Typhi strains, such as those already identified in the DNA samples of Kerameikos, may offer in the future some reasonable explanation for the differences in clinical features observed between the Plague of Athens and the present day form of typhoid fever (Black et al. 1985).

Nine criteria for validating ancient DNA studies have been proposed (Cooper and Poinar 2000): (1) a physically isolated work area, (2) control amplifications, (3) appropriate molecular behaviour of PCR products, (4) reproducibility of results, (5) cloning of amplified products, (6) survival of associated human DNA remains, (7) independent replication by sequencing in independent laboratories, (8) biochemical preservation studies of DNA, and (9) quantitation of copy number of target DNA using competitive PCR. So far, the first seven criteria have been met for the Plague of Athens, while the remaining two will follow in subsequent studies.

Future prospects for research include the investigation of more Plague victims from Kerameikos, both for *S. enterica* Typhi sequences and those of other candidate pathogens. In addition, a major challenge is the precise genetic characterisation of the ancient strain of the typhoid fever agent, which might lead not only to understanding of its aggressiveness 24 centuries ago, but also to possibly develop animal models of typhoid fever, an important tool to research and combat this disease.

Studying the historical aspects of infectious diseases can be extremely useful in several disciplines. This study sheds light on one of the most debated enigmas in medical history and therefore may contribute to many scientific fields. Archaeology, history, microbiology, palaeopathology, certain fields of medicine, anthropology and even genetics, molecular biology and biology of evolution are clearly implicated in such matters and can benefit from relevant studies.

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