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# FEATURES OF THE STUDY OF ANCIENT PATHOGENS

**Abstract.** Infectious disease have affected humans throughout their evolution, both during acute pandemic events and through persistent morbidity and mortality. Recent advancements in the field of ancient genomics have increased our understanding of the infectious disease history and pathogen evolution though time. For the article, we used more than 300 ancient teeth samples from Central Asia, spanning the time period between the 4th century BCE and the 4th century CE, to address questions related to pathogen presence in past populations and microbial evolution through time. It focused on non-targeted metagenomic approaches for the detection of ancient pathogens in ancient human remains. Additionally, the article describes a specific case study of paleo-DNA screening for the plague pathogen, *Yersinia pestis*, which is known to have afflicted human populations since the Neolithic period, has caused immense mortality during the Middle Ages in Europe and continues to be prevalent across Central Asia today.

**Key words:** ancient DNA, pathogens, plague, pla-assay.

## Introduction

Humans have been exposed to a wide range of microorganisms throughout their history, and such interactions are likely to have influenced human evolution and health through time. To date, it is eastimated that about a trillion species of microorganisms exist[1], the vast majority of which are unknown. Of these, about 1,400 are know to be pathogenic to humans[2,3]. According to the observed virulence and host adaptation and the natural history of these interactions remains unknown.

Using new high-performance methods, bioinformatical analysis and the expansion of publicly available data sources leads to new evidence of the evolution of numerous pathogens associated with humans [4]. For example, recent studies have shown the existence of plague bacteria (*Yersinia pestis*) in humans during the early Bronze Age, more than 3000 years prior to the disease being recorded in historical sources[5]. In addition, some scientists recognized seals as a probable source of human tuberculosis [6], while others noted a high level of genome conservation in leprosy for 1000 years of its evolution[7].

Given its high potential in deciphering pathogen evolution through time, research on ancient pathogens has received a lot of attention during the past decade, and techniques for optimized data retrieval and authentication are continuously being developed [8].

At the moment there are many theories about the occurrence of the plague, finding the most ancient, you can explore and give answers to many questions of interest [9, 10]. Starting during the 1990s, some of the pioneering research on the evolution of *Y. pestis* reveiled itsclose genetic relationship to*Y. pseudotuberculosis*, which led to the conclusion of their recent divergence, however, without indicating the time and place exactly when and where it happened [11].Such findings were followed by more published work regarding *Y.pseudotuberculosis* as a direct ancestor of the plague bacterium [12].

More recently, the study of infectious disease history and evolution has benefitted from the collaboration between different disciplines, including those of microbiology, history, anthropology,archaeology, as well as that of paleogenetics, which involves the use of ancient DNA to decipher the riddles of the

past.Some of the main question this field has dealt with involves the identification of the causesof ancient epidemic or pandemic events, and the evaluation of pathogen presence in human populations through time.As such, new and optimized techniques that enable the study of ancient DNA from archaeological material and computational-tools used for evolutionary assessment enable us to reconstruct the history of ancient epidemics and look for precursors of modern infectious disease pathogens.

In this study, we present preliminary results *Y. pestis*-specific screening screening of 333 human dental samples from various archaeological contexts using a previously published qPCR assay [15].

## 2. Materials and methods

The most frequently used specimens for the analysis of pathogen DNA in archaeological remains is teeth. As it turned out, the causative agents of infectious diseases accompanied by bacteremia, such as, plague causative agents, typhus, etc., are transported via the blood flow to the teeth's (pulp) inner portion. After the death of the diseased, the DNA of these pathogens is maintained in the dental pulp. Dental sampling has therefore been effective in acquiring complete genomes from ancient bacteria such as *Y. pestis*, Borrelia recurrentis and *Salmonella enterica*, as well as ancient viruses such as the hepatitis B virus (HBV) and the human parvovirus B19 [8].

*Sample collection.* The sample collection was performedatthree locations. First, 136 ancient teeth from Kazakhstan and 3 teeth from Bashkiria were collected from the Institute of Archeology, Almaty, Kazakhstan. In addition, 187teeth from Central Asia were collected from the Scientific Institute and Museum of Anthropology, Lomonosov Moscow State University, in Moscow,spanning the time period between the 4th century BCE and the 4th century CE. Finally, 7 tooth samples were collected from the Peter the Great Museum of Anthropology and Ethnography (the Kunstkamera), St. Petersburg.

*Tooth sampling.* All manipulation of teeth sampling have done in the clean room and using sterile materials in the biosaty cabinet to avoid carry over contaminations, at the Max Planck Institute for the Science of Human History in Jena, Germany. The tooth were separated into two parts with a drill blade: tooth crown and tooth root, where kept the root to store for back-up DNA or radiocarbon and other. It was drilled out 40–150 mg of dental pulp [13].

*Extraction of ancient DNA.* All ancient DNA lab work was performed at the dedicated facilities of the Max Planck Institute for the Science of Human History (MPI-SHH) in Jena, Germany from 17th of February till 15th of May, 2019. 333 teethwere sampled and used for DNA extraction. For the DNA extraction we useda published protocol, optimized for the extraction of short DNA molecules, therefore, tailored for the extraction of aDNA [14].

*Pla-assay*.All DNA extracts were screened using a qPCR assay designed for the amplification of the *pla* gene in *Y. pestis*, present on the multi-copy and species-sepcific plasmid pPCP1. The assay was carried out using a previously published protocol [15].

## 3. Results and discussion

All samples were extracted and tested for *Y.pestis* by qPCR at the Max Planck Institute for the Science of Human History (MPI-SHH) in Jena, Germany. Using approximetly 50 mg of powederised dental pulpfrom human teeth,theextracted DNA was screening for the presence of *Y. pestis* using a qPCR assay targeting the *pla*gene of the *Y. pestis*-specific plasmid pPCP1. All reactions were carried out onLightCycler® 96 (Roche, Mannheim, Germany). Master-mix preparation is presented in Table 1.

Cycling conditions started with preincubation step at 95°C for 12 min. This was followed by 3 step of amplification, at 95°C for 30 sec, an assay specific annealing temperature for 30 sec, and 72°C for 30 sec, ending with an elongation step at 72°C for 30 sec. And then melting at 95°C for 21 sec,annealing temperature 60°Cfor 30 sec, and 90°C for 1 sec. Final step at 37°C (Table 2).

Table 1 – Pla-assay Master Mix

| Components | stock concentration | Final concentration | units | 1X volumes (ul) |
|---|---|---|---|---|
| 10X PCR buffer 2 | 10 | 1 | X | 2 |
| 25mM MgCl2 | 25 | 2.5 | mM | 2 |
| 25mM dNTP mix | 25000 | 250 | uM | 0.2 |
| DMSO | 100 | 5 | % | 1 |
| 10mg/ml BSA | 10 | 0.75 | mg/ml | 1.5 |
| EVA green | 20 | 1 | X | 1 |
| pla_Ef | 10000 | 300 | nM | 0.6 |
| pla_Er | 10000 | 300 | nM | 0.6 |
| Amplitaq gold | 5 | 0.05 | u/ul | 0.2 |
| ddH20 | | | | 8.9 |

Table 2 – Cycling conditions for pla-assay.

| | **Temperature** | **Time** |
|---|---|---|
| Preincubation | 95C | 12min |
| 3 step Amplification | 95C | 30sec |
| | 61C | 30sec |
| | 72C | 30sec |
| Melting | 95C | 21sec |
| | 60C | 30sec |
| | 90C | 1sec |
| Cooling | 37C | |

QPCR assays were carried out on a LightCycler 96 platform (Roche, Mannheim, Germany). Quantification of *pla*-qPCR assays was possible by determination of the copy numbers per reaction by generating a standard curve using synthetic oligonucleotide constructs. Standard curve presented in figure 1.

The concetration of standards that we used presented in table 3.

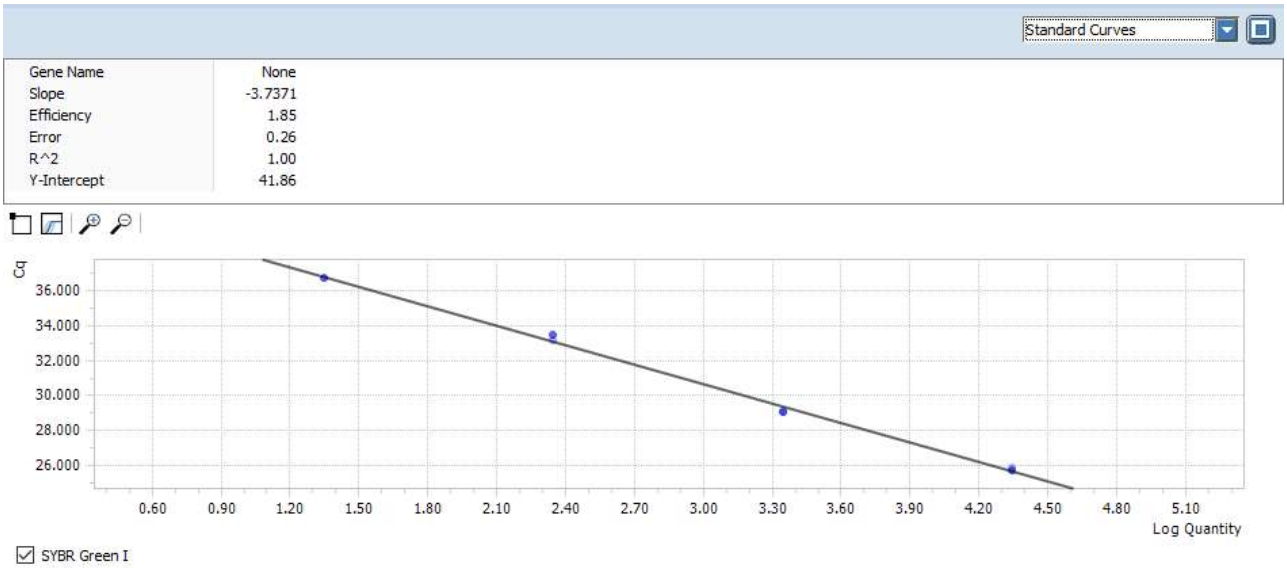


Figure 1 – Standard curve from qPCR

The positive sample was subjected to melting curve analysis together with standards. In summary, overlapping melt curves of the amplicon in each positions demonstrated temperature homogeneity and equal treatment of sample and standard. In figure 2and figure 3 blue colores presented standards that we used and green is our sample which is potentially positive

Data analysis was performed using the LightCycler 96 (Roche, Mannheim, Germany). The green line showed our positive result for plague.

Table 3. Standards and blanks for pla-assay.

| Position | 1 | 2 |
|---|---|---|
| A | 2,23E+004 | 2,23E+004 |
| B | 2,23E+003 | 2,23E+003 |
| C | 2,23E+002 | 2,23E+002 |
| D | 2,23E+001 | 2,23E+001 |
| E | 2,23E+000 | 2,23E+000 |
| F | 2,23E-001 | 2,23E-001 |
| G | blk | |
| H | blk | |

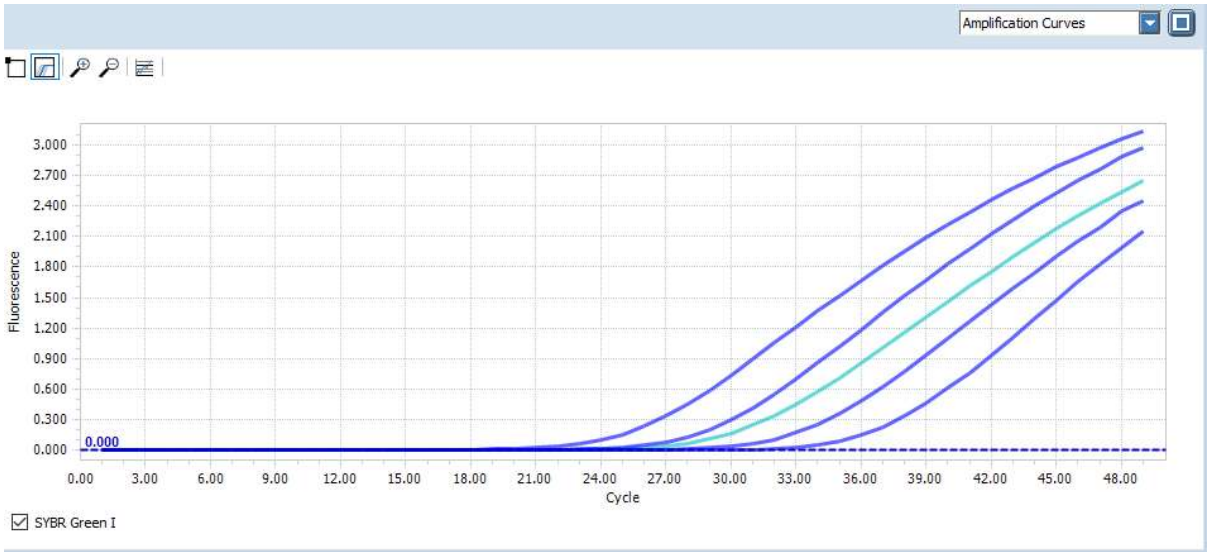


Figure 2 – Amplification curves from pla-assay.

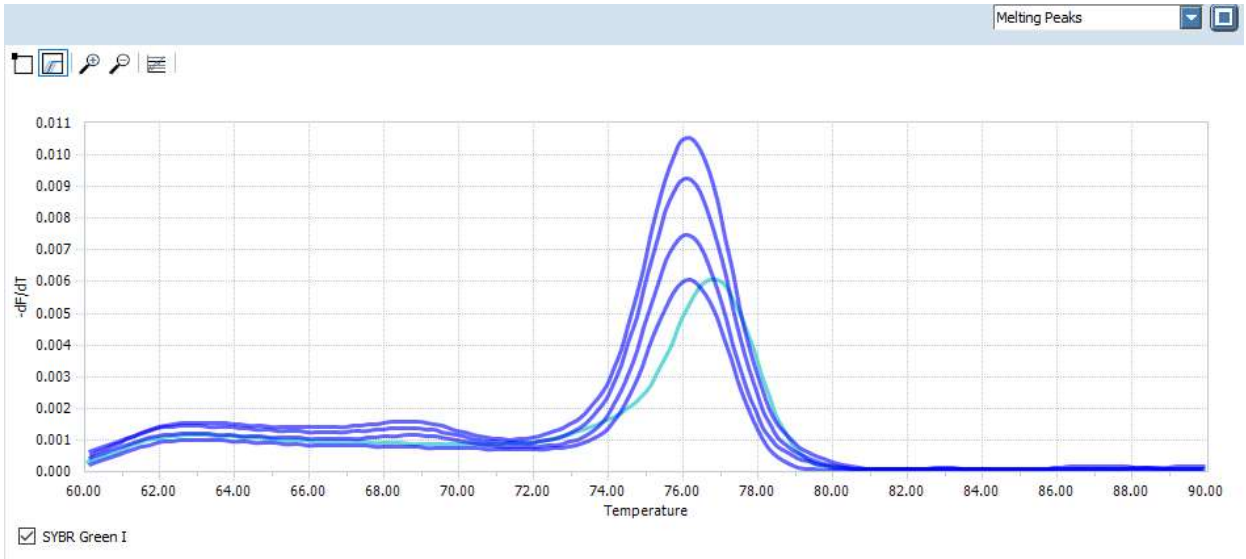


Figure 3 – Melting peaks from pla-assay.

**Conclusion**

For many ancient samples, the content of endogenous DNA is very low (<1%), therefore, recently attempts have been made to improve access to the DNA of interest through the development of new sampling, DNA isolation and library preparation protocols as well as through hybridization enrichment techniques.

Using qPCR we detected some potentially-positive results for plague,through such results can not be confirmed without further sequencing.Future analyses of the potentially-positive samples using next generation sequencing, will enable an assessment of the current results and potentially enable the in depth study of the evolution of *Y. pestis*.

**Conflist of interest**

All authors have read and are familiar with the contents of the article and have no conflict of interest.

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## ЕЖЕЛГІ ПАТОГЕНДІ МИКРОАҒЗАЛАРДЫ ЗЕРТТЕУ ЕРЕКШЕЛІКТЕРІ

**Аннотация.** Жұқпалы аурулар адам популяциясына бүкіл эволюция кезеңінде, әсіресе пандемия арқылы тұрақты сырқаттанушылық пен өлім арқылы әсерін тигізіп келеді. Археогенетика саласындағы соңғы жетістіктер уақыт өте келе жұқпалы аурулар тарихы және патогендік эволюция туралы түсінігімізді арттырды. Мақала үшін біз б.э.д. IV ғасырдан б.з. IV ғасырға дейінгі уақытты қарастыра, Орталық Азиядағы ежелгі тістердің 300-ден астам үлгілерін қолданып, бұрынғы популяциялардағы патогеннің болуы және уақыт өткен сайын микробтардың эволюциясы туралы сұрақтарға жауап береміз. Ол ежелгі адамның қалдықтарында ежелгі патогенді метагеномдытәсілдерімен анықтау арқылы жасалынды. Бұған қоса, мақалада неолит дәуірінен бері адам популяцияларымен ауырған, Еуропада орта

ғасырларда өте көп өлімге әкеліп соқтырған және бүгінгі күнде Орталық Азияда кең таралған аурудың бірі обаның қоздырғышы, *Yersinia pestis*палео-ДНҚ үлгілерін скрининг арқылы нақты жағдайын сипаттайды.

**Түйін сөздер:** ежелгі ДНҚ, патогендер, оба, pla-талдау.

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## ОСОБЕННОСТИ ИССЛЕДОВАНИЯ ДРЕВНИХ ПАГЕННЫХ МИКРООРГАНИЗМОВ

**Аннотация.**Инфекционные заболевания поражали людей на протяжении всей их эволюции, как во время острых пандемических событий, так и в результате постоянной заболеваемости и смертности. Последние достижения в области археогенетики расширили наше понимание истории инфекционных заболеваний и эволюции патогенов. Для этой статьи мы использовали более 300 образцов древних зубов из Центральной Азии, охватывающих период времени между 4-м веком до нашей эры и 4-м веком нашей эры, для решения вопросов, связанных с наличием патогенных микроорганизмов в прошлых популяциях и эволюцией микробов. В статье описаны не целевых метагеномные подходы для обнаружения древних патогенов в древних останках человека. Кроме того, приведен конкретный пример скрининга палеоДНК образцов на возбудитель чумы *Yersinia pestis*, который, как известно, поражал человеческое население со времен неолита, и вызвал огромную смертность в средние века в Европе и продолжает преобладать в Центральной Азиисегодня.

**Ключевыеслова:** древняя ДНК, патогены, чума, pla-анализ.