REVIEW

Palaeopathology: scope and opportunities in India

Satya Prakash[a*] and Niraj Rai[b*],

[a] CSIR- Centre for Cellular and Molecular Biology, Hyderabad

[b] Birbal Sahni Institute of Palaeosciences, Lucknow

*Corresponding author e-mail: nirajrai@bsip.res.in; satya.science@gmail.com

ABSTRACT

Life on the earth is full of secrets. The origin of diseases in humans, body's susceptibility to pathogens and initiation of diseases due to the environmental influence are few among those many secrets that remain unrevealed till date. Recent advancements in the technology have linked history with science by maneuvering successful use of scientific methods and tools in deciphering and understanding historical events. Thus, this connection paves an important means that can be utilized for studying molecular factors responsible for a disease or an epidemic that happened in the past.

The development of high-resolution microscopy, high throughput sequencing technology coupled with strong computational methods can be extremely helpful in revealing host-pathogen relationship that existed or began to nurture in the past. Palaeopathology is an interdisciplinary science which involves robust archaeological expertise along with high end medical and molecular proficiency. Palaeopathology is the study of past diseases, pathogens and host-pathogen relationship using archaeological samples.

KEYWORDS: Palaeopathology; molecular archaeology; host-apthogen interaction; ancient DNA

Citation: Prakash S and Rai N. Palaeopathology: scope and opportunities in India. Polymorphism 2019;3:7-14.

INTRODUCTION

Ancient DNA (aDNA)

DNA isolation from archaeologically important biological samples is an emerging method cum tool to study the relationship between a species which lived in the past and the contemporary population (Hofreiter et al., 2001). However, the high level of degradation in aDNA, cytosine deamination and contamination of aDNA due to its casual but frequent contact with modern DNA are standing as the major hurdles in aDNA research. The fact that DNA molecules present in the skeletal and paleontological remains are extremely low than that of modern biological specimens, makes this field even more vulnerable to hypothesize any quantifiably robust study (Allentoft et al., 2012; Renaud et al., 2015). Interestingly, with the advent of cutting edge high throughput sequencing technologies, now it is possible to access the real aDNA and understand its presence in a sample and one can determine the damage patterns of aDNA in the mixture of total DNA (Jonsson et al., 2013). To enrich the quantity of aDNA, researchers use unique methods which are based on DNA adaptor ligation at the ends of DNA molecules employed during library preparation (Dabney et al., 2013). Using high-performance computing and statistical analysis, nucleotide misincorporation and exogenous contamination in aDNA can be identified from high throughput sequencing data (Jonsson et al., 2013). Besides, the reconstruction of the complete ancient genome and its analysis against modern genome is now possible with the introduction of high throughput next-generation sequencing (NGS) (Schubert et al., 2014).

Going back to history, aDNA started with Quagga, a zebra-like horse family member that became extinct in 1883. In 1984, a study published the sequencing of DNA isolated from the dried muscle of a museum specimen of Quagga (Higuchi et al., 1984). It was the first study on aDNA. Till now, several studies have been conducted in this field to disclose the secrets of human evolution, migrations, food habits (Braun et al., 2010) and domestication (Librado et al., 2017). In this continuation, aDNA study on the bone of a female died 90,000 years ago discovered in Siberian cave showed half Neanderthal and half Denisovan (Slon et al., 2018). It was concluded as the offspring of a Neanderthal mother and a Denisovan father.

aDNA studies in India

In the year 2014, three bone samples were excavated from St. Augustine Convent, a world heritage site in Goa. All the three bone relics were taken for mtDNA (mitochondrial DNA) analysis and sex identification using Amelogenin marker. The result of the analysis showed that one of the bone was related to a female with U1b haplogroup which is not found in Indian sub-continent. Further investigations with 30 contemporary Georgian samples made it clear that the bone sample QKT1 was likely of Queen Ketevan of Georgia (Rai et al., 2014). This was the first ever scientifically published aDNA study from India. Thereafter, many ancient graves were excavated in and around India to study human evolution and migration to and from India. The following are few such case studies.

Parsis, one of the minority communities in India, who also represent a minuscule population group in the world, have been practicing the tradition of cremating their dead bodies by exposing them to the natural environment in a well type of structure called Dhokama. In the quest of aDNA study, 21 samples were collected from Sanjan Dhokama in Gujarat. After analyzing mtDNA and Y chromosome from 21 ancient bone samples along with 174 contemporary Parsi individuals from India and Pakistan, it was found that Parsi population, mainly males migrated to India in the 7th century AD. Y chromosomal analysis showed the modern Parsi population with a higher frequency of Middle-Eastern-specific lineages, while mtDNA analysis showed the Parsi community closer to the Indian and Pakistani population (Chaubey et al.,

2017). Thus, it can be inferred that in the early stages of migration, they mated with local females.

Roopkund lake is situated at 5029 meters above sea level in Chamoli district of Uttarakhand. The lake remains frozen most of the time and melts only for a week during summer. Once it melts, one can see scattered human skeletons all-around the lake. Carbon dating shows that the bones are from 800 to 850 AD. Further investigations are going on to reveal their secrets, like who those people were, where from did they came, what were they doing at such a high altitude, and how they died together in such a single instance.

In the context of aDNA studies in India, many excavated samples from Harappan sites in Rakhigarhi, Farmana, and Kunal, Haryana are under investigation at present. Some samples are being excavated at Inamgaon in Maharastra, Sanauli in Uttar Pradesh, Rohtas in Bihar, Pattanam in Kerala, Vadnagar in Gujarat and many other sites. Work is going on not only on humans but also on animals and cereals too, which can reveal the domestication pattern of different animals and provide clues about the diet habits/patterns of humans and animals in the past. The first ancient DNA laboratory in India was established at the Centre for Cellular and Molecular Biology, Hyderabad under the leadership of Dr. Lalji Singh. Studying aDNA in India has been accelerated and furthered after the establishment of a new aDNA Laboratory at the Birbal Sahni Institute of Palaeosciences, Lucknow.

Palaeopathology

Palaeopathology is a medical discipline of aDNA (Fernandez, 2012). It may seem to be a new terminology to Indian science, but the study of diseases in archaeological remains (either human or animal) started in 1929 (Jane Buikstra, 2012). Palaeopathology is the study of ancient diseases, ancient host-pathogen relationship, ancient pathogen genomes and what made humans susceptible to a particular disease. If any ancient disease is still in existence, the Palaeopathology as a discipline, allows us to do a comparative study of ancient and modern diseased conditions along with the investigation of ancient and modern pathogen genomes (Spigelman et al., 2015). Bones are the primary samples for Palaeopathological studies, but sometimes preserved soft degraded tissue remains can be of great help (Fernandez, 2012). These degraded tissues may be a good source of ancient DNA and protein. The techniques of studying Palaeopathology are microscopy, immunohistochemistry and molecular biology (Fernandez, 2012) - especially ancient DNA (aDNA) studies. It is an interdisciplinary science where anthropologists play an important role, while a substantial amount of medical training and modern expertise of medical professionals would be pivotal. Especially, while carrying out comparative studies on diseases involving both modern and ancient forms, competent guidance of medical professionals is must.

Often, skeletal and dental records have been very useful in understanding the lifestyle of a person as bones are susceptible to various stresses during their lifecycle that can alter their shape, size, consistency and development. Consequently, the observation of human remains together with medical knowledge enables the observer to diagnose acute and chronic lesions that were shaped by genetic, infectious, neoplastic, joint, traumatic or metabolic processes. We explore the knowledge of morphological changes in bones after infection and using this knowledge, we aim to reconstruct the genetic makeup and virulence of pathogens who have infected our ancestors in the past.

The excavation of skeletons with morphological indications of TB, and the likely presence of Mycobacterium in the bones at the time of death has made TB an attractive target for aDNA studies. The first report of *M. tuberculosis* in aDNA detection (Jaeger et al., 2012; Muller et al., 2014) has been followed by a substantial number of publications describing the use of the polymerase chain reaction (PCR) to detect MTBC in human

bones and teeth (Bouwman et al., 2012; Muller et al., 2014).

Similarly, Leprosy, which is an infection caused by the pathogen *Mycobacterium leprae* has been prevalent since age immemorial. References of its existence have been inscribed in the historical texts in India and elsewhere (Dharmendra, 1947; Hulse, 1976). Investigations on *M. leprae*'s evolutionary history have elucidated the past phylogeography and diversity of the leprosy bacillus in Europe. Recently sequenced medieval *M. leprae* genomes reveal the presence of at least two distinct *M. leprae* branches in medieval North-western Europe (Schuenemann et al., 2013). Furthermore, the data indicate a high level of genetic conservation that happened for *M. leprae* during the last 1000 years of its existence and evolution. It also appears that there exists a close relationship of a group of late medieval strains with contemporary strains present today in the South-western USA (Schuenemann et al., 2013), which are infecting humans and armadillos (Truman et al., 2011) as well as red squirrels in England (Avanzi et al., 2016). The earliest accepted written record of leprosy is in the Sushruta Samhita, an old Indian text on medicine and surgery dated around 600 B.C.E. (Dharmendra, 1947), but the exception is that only a limited number of probable cases are mentioned therein. However, based on paleopathological evidence available so far, the oldest evidence of leprosy from India dated around 2000 B.C.E. (Robbins et al., 2009).

There exist many controversies surrounding leprosy, which is one of the oldest recorded diseases of humankind. The origin and spread of its main causative agent, *Mycobacterium leprae* during ancient days remain unknown till date, although many attempts have been made to reconstruct its past from historical and archaeological sources. Analysis of ancient *M. leprae* genomes reconstructed using the aDNA isolated from the archaeological remains can contribute greatly towards understanding the origin and evolution of this pathogen. With a new set of ancient *M. leprae* genomes from Europe, a so far unrecognized past diversity has been traced back, which places Europe as a key region for the early spread and worldwide dissemination of leprosy. The results hint to the potential dynamic changes in the prevalence of different *M. leprae* strains in Europe during antiquity and highlight the need to study ancient pathogen genomes in order to better understand our past.

Coming to the Cholera prevalence in India, Cholera continues to remain an important public health concern in developing countries. Globally, the true number of cholera cases is known to be much higher than reported. The discrepancy results from underreporting and other surveillance system limitations, including inconsistencies in case definitions and lack of standard vocabulary. Seven distinct pandemics of cholera have occurred since the onset of the first pandemic in 1817 (Colwell et al., 2003; Kaper et al., 1995). Except for the seventh pandemic which originated in Indonesia, six of the pandemics arose from the Indian subcontinent, usually from the Ganges Delta region, and reached to other continents (Kaper et al., 1995). Besides the above, the Indian context of plague infection is not well studied.

METHODOLOGY AND TECHNIQUES

Direct evidence for pathologies and former treatments can be obtained from macroscopic examination of human remains. Skeletal and dental records are very useful for this purpose as bones are vulnerable to various internal and external stresses encountered by a body during its lifecycle and left evidence within itself in the form of deviations in its shape, size, consistency, and development.

To extract DNA from the infected ancient human's skeletal remains, around 30–50 mg bone powder is generally used. A silica purification protocol is more efficient method, which is in practice with little modifications.

DNA aliquots of up to 20 µl from each sample will be converted into double-stranded genome libraries (Meyer et al., 2010). The adapter-ligated fragments will be quantified through a quantification assay using the primers IS7 and IS8 (Meyer et al., 2010), and the SYBR Green qPCR Kit. Following established protocols (Kircher et al., 2012; Meyer et al., 2010), sample-specific indexes will be added in the next step to both library adapters via amplification to create double indexed libraries.

For genome-wide enrichment and sequencing, additional libraries will be prepared from 30 to 50 µl aliquots of all DNA extracts according to the methods described elsewhere (Kircher et al., 2012; Meyer et al., 2010) with one modification. One additional step - the treatment of all extracts and blanks with uracil-DNA glycosylase (UDG) and endonuclease VIII- is included into library preparation to avoid potential sequencing artifacts caused by the characteristic ancient DNA damage profile produced by the deamination of cytosine to uracil over time (Briggs et al., 2010). For all indexed libraries, a subsequent amplification will be performed as detailed by Schuenemann and colleagues (Schuenemann et al., 2013).

Enrichment and sequencing for sample screening

Depending upon the aim and type of the samples, questions and objectives, the double-stranded libraries of the corresponding ancient pathogens will be screened for *M. leprae*, *M. tuberculosis*, *Yersinia pestis* & *Vibrio cholera*. We will prepare the targets for DNA enrichment and convert into bait DNA using Long Range PCR products as described previously (Schuenemann et al., 2013). Following the bead enrichment protocol by Maricic and colleagues (Maricic et al., 2010), hybridization of the amplified libraries pooled in an equimolar amount to the DNA bait will be carried out. Subsequently to the bead enrichment, the libraries will be sequenced on an Illumina HiSeq 2500 or equivalent platform using a paired-end dual index run.

Genome-wide enrichment and sequencing

In the case of *Mycobacterium leprae*, the UDG treated libraries will be enriched genome-wide with two rounds of hybridization capture following the protocol detailed before (Hodges et al., 2009). The design of the 1 million Agilent SureSelect arrays will be used in the study, which was described previously.

Scope of Palaeopathology in India

India is home to incredible biodiversity, which remains under-studied in the genetic context. aDNA is starting to revolutionize the study of how present-day diversity comes to the form and how it is still continually evolving. aDNA tool has been extensively used in various parts of the world such as North America, Europe and Australia, but remains yet to be used effectively in the Indian context. aDNA is essentially a time capsule of the past genetic diversity and the best can be used in consort with modern-day DNA datasets to provide a fine-scale understanding of how life evolves as well as the role of the environment in evolution. Using Archaeological samples, one can understand the long-term evolutionary changes in pathogens and their pathogenesis during the course of human civilization in Indian sub-continent for e.g. genetic variation in pathogens i.e *Mycobacterium Tuberculosis*, *Mycobacterium leprae*, *Yersinia pestis* and *Vibrio cholera* from infected human skeletal remains and soil sediments.

The current work on relics in India has mainly focused on human evolution, migration, and domestication while investigating from the paleopathology point of view has not been paid heed. For example, the malaria parasite is one of the major health issues in India (Das et al., 2012). But the history of the malaria parasite and human interaction is still unclear. Using palaeopathological tools, we can study these types of host-pathogen relationships that established in the past and compare them with the present day scenario to better understand human diseases.

The studies of pathogens' genetic material in the past, their evolution with time and the root of interaction with humans may help in case of bacterial diseases like tuberculosis and leprosy (Spigelman et al., 2015). India is rich in its collection of human remains from the past. Many universities have their own collections in their respective museums. These samples can easily be made accessible to researchers for palaeopathological studies

CONCLUSION

The study of all the above-mentioned diseases has a long history and most probably has advanced rapidly over the last 40–50 years with the development of methods, and particularly ancient pathogen DNA analysis. While highlighting that palaeopathology has close interactions to the evolutionary medicine, it focuses on three 'case studies' that illustrate the close interaction people have had with their environments and how that had impacted their health. Tuberculosis, leprosy, plague, and cholera, in particular, have remained omnipresent diseases throughout the thousands of years of our existence. Ancient DNA methods are now allowing us to explore how strains of the bacteria causing TB, Leprosy, plague and cholera have changed with time.

How do the environment (climate) and humans come together over time to impact on the food and water resources in India? This question may be addressed by investigating the evolutionary trajectory and drug resistance mechanism of the bacteria *Vibrio cholera*, the causative agent of cholera, as well as by studying how mycobacterium virulence evolved over a couple of thousand years. Furthermore, the ancient genome of *Y. pestis* will help us in understanding the origin and spread of plague on a global level. Retrieved ancient DNA sequences will be compared to modern-day counterparts of these species to understand their evolutionary trajectories that can trace the origin of the pandemics and the pathogen's resistance acquisition mechanism over time. Ultimately, palaeopathology has the potential to contribute towards a multitude of fields including public health and epidemiology, vaccine development strategies, evolutionary biology, sanitation, agricultural policies and life conservation in India.

Acknowledgments

The authors would like to thank the Birbal Sahni Institute of Palaeosciences, Lucknow for providing the necessary infrastructure for their studies on ancient DNA.

Conflict of interest statement

The author has declared that no competing or conflict of interests exists.

Authors' contributions

Satya Prakash and Niraj Rai conceived, designed and wrote the manuscript.

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