Ancient-Pathogen Genomics: Coming of Age?

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ABSTRACT The potentially debilitating zoonotic disease brucellosis is thought to have been a scourge of mankind throughout history. New work by Kay et al. [mBio 5(4):e01337-14, 2014] adds to evidence for this by exploiting the huge advances in next-generation sequencing technology and applying shotgun metagenomics to a calcified nodule obtained from a 14th-century skeleton from Sardinia. While not the first DNA-based confirmation of *Brucella* in medieval DNA samples, Kay et al.'s study goes much further than previous reports based on single gene fragments in that it allows a full-genome reconstruction and thus facilitates meaningful comparative analysis of relationships with extant *Brucella* strains. These analyses confirm the close relationship of the genome to contemporary isolates from the western Mediterranean, illustrating the continuity of this lineage in the region over centuries. The study, along with recent studies characterizing other ancient-pathogen genomes, confirms that shotgun metagenomics offers us a powerful tool to fully characterize pathogens from ancient samples. Such studies promise to revolutionize our understanding of the nature of infectious disease in these materials and of the wider picture of the emergence, evolution, and spread of bacterial pathogens over history.

Brucellosis remains one of the world's most significant zoonotic diseases still widely distributed globally in both animals and humans, with effective control and/or eradication achieved in only some of the most developed countries (1). While over 500,000 new human cases a year are reported globally (2), the nonspecific clinical picture of brucellosis, as well as low disease awareness and poor diagnostic capabilities in many parts of the world, means that this figure is likely a gross underestimate. Ten species of *Brucella* are now recognized as members of a largely genetically highly conserved group (3), but the most economically significant are *Brucella abortus* (responsible for bovine brucellosis), *Brucella melitensis* (brucellosis of small ruminants), and *Brucella suis* (swine brucellosis). All of these three species are also highly pathogenic for humans, with *B. melitensis* thought to be responsible for the largest human disease burden. Human infection usually occurs as a result of occupational exposure (abattoir workers, farmers, hunters, and veterinarians) or as a result of consumption of unpasteurized dairy products, reflecting excretion of the organism in the milk of domesticated animals.

Brucellosis in animals is associated with abortion, retained placenta, orchitis, or epididymitis, and abortion causes a massive release of the organism into the environment. In humans, initial symptoms are nonspecific and can include an undulant fever, tiredness, night sweats, appetite and weight loss, and headaches, all of which, without treatment, can become severe and debilitating. Left untreated, the infection can become chronic – in such cases osteoarticular manifestations, such as arthritis, sacroiliitis, or spondylitis, are frequent forms of localized disease. Osteomyelitis caused by brucellosis most frequently affects the lumbar and lower thoracic vertebrae, and an important differential diagnosis in cases of osteomyelitis or septic arthritis is tuberculosis (4).

How long humans have been living with brucellosis is unclear. Being an insidious and often nonspecific disease that debilitates, rather than kills, there are no dramatic epidemics in historical records, unlike for diseases such as plague. Writers have tried to link outbreaks of disease reported in ancient literature and historical records, such as in ancient Greece (5th century BC), with brucellosis based on the descriptions (5), and it has been suggested that brucellosis was endemic in Roman society (6). More-direct evidence has come from a number of paleoanthropological studies that have identified vertebral lesions consistent with brucellosis from Early Bronze Age samples in the Middle East, from skeletons of adults fleeing the eruption of Vesuvius in AD 79, and from later European samples (6). Further, it has recently been hypothesized that the macroscopic, microscopic, and radiological appearance of lytic lesions in lumbar vertebrae of an australopith (*Australopithecus africanus* Stw 431) from the Late Pliocene site of Sterkfontein in South Africa is consistent with skeletal pathognomonic characteristics of brucellosis (7). This observation raises the possibility of the presence of brucellosis in hominids 2.3 to 2.5 million years ago.

The above-described studies all rely on potentially challenging differentiations of lesions characteristic of brucellosis from those of other infectious diseases, such as tuberculosis or staphylococcal spondylitis, which can cause superficially similar, though less discriminate, lesions. The earliest laboratory-confirmed cases, thought to date from between the 10th and 13th centuries, have been described from the ancient Albanian city of Butrint (8). Here, lesions on thoracic and lumbar vertebrae of skeletal remains of two adolescent males were identified. While pathologies suggested brucellosis or tuberculosis, genetic screening of skeletal samples for tuberculosis was repeatedly negative. However, two *Brucella*-specific DNA markers, IS*711* and *bscp31*, could be amplified by PCR from the affected vertebrae but not from unaffected individuals or control samples. Subsequent sequencing confirmed the presence of IS*711* and provides strong direct evidence that brucellosis has been endemic in this area, which still reports some of the highest incidences in Europe today, since at least the Middle Ages.

The study described above is typical of those undertaking mo-

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lecular examination of ancient-pathogen DNA until recently (9). Since the emergence of the use of genetics in paleomicrobiology some 20 years ago, studies have relied largely on PCR amplification of small fragments of DNA from historical material. While much has been gleaned from such studies, they are limited in many respects, particularly by concerns about cross-contamination with modern material, either in the laboratory or environmentally, and by the limitation that the small fragments of DNA obtained restrict the ability to place ancient DNA in context with modern bacterial strains of the same group. However, paleomicrobiology, at least for some microorganisms, now promises to be revolutionized by the emerging field of ancient-pathogen genomics driven by the astronomical pace of advancement in next-generation sequencing technologies.

The field has been driven largely by application to three organisms, *Yersinia pestis*, *Mycobacterium leprae*, and *Mycobacterium tuberculosis*, for which pertinent samples are more easily identifiable through historical records or pathological markers; in addition, the biological properties of these organisms mean that genetic material may be better preserved. Given the historical impact of the Black Death, caused by *Y. pestis*, application of paleomicrobiology to provide concrete evidence of the causative agent and to understand the evolution of the organism has been of substantial interest. In 2011, the genome of *Y. pestis* was reconstructed at 30-fold coverage from the teeth of Black Death victims from a London graveyard dated to the mid-14th century (10). Phylogenetic analysis at the time showed the strain to be close to the ancestral node of all strains that cause human disease, suggesting that contemporary *Y. pestis* epidemics originated in the medieval period. Compared against modern genomes, no unique derived positions were detected in the medieval genomes, indicating that the perceived increase in virulence associated with the Black Death may not reflect bacterial genotype but other factors, such as environmental or social changes, the impact of concurrent infectious disease, vector dynamics, or changes in host susceptibility. However, an alternative explanation that cannot be excluded is that the virulence of the organisms may be affected by areas of the genome other than those currently recognized (9). This reflects the fact that this study, as well as a further recent study confirming that the *Y. pestis* associated with the plague of Justinian in AD 541 to 543 is an independent emergence into humans from rodents that may now be extinct (11), utilizes capture techniques in which modern sequences are used to drive hybridization and "enrich" for DNA of interest. Thus, inherent weaknesses of these approaches are that one finds only what one is looking for and that they may fail to detect sequences present in ancient strains but absent in contemporary ones.

Shotgun metagenomics addresses the above issue by allowing an unbiased sequencing *en masse* of DNA extracted from a sample without target-specific amplification or capture. In 2013, what was believed to be the first full *de novo* assembly of a genome via shotgun sequencing alone representing an ancient pathogen from skeletal material held at ambient temperature was reported (12). From one sample, the tooth of a woman who died in a leper colony in Denmark in the 1300s, the finding that an astonishing 40% of sequence reads mapped to *M. leprae* allowed the *de novo* assembly of an *M. leprae* genome with 100-fold coverage and with 169 contigs separated by gaps corresponding to repetitive regions. This genome, along with four others obtained following enrichment by traditional capture approaches, was compared with modern *M. leprae* strains, revealing remarkable conservation over a millennium. These data suggested that the decline in leprosy in 16th-century Europe likely did not reflect a loss of virulence of the pathogen; they also suggested a European origin for leprosy in the Americas and showed that strains currently circulating in the Middle East likely originated in medieval Europe. The authors concluded that the study implied the real prospect that the prehistoric origins of *M. leprae* might be traced by *de novo* genome assembly. At around the same time, a shotgun metagenomics study applied to mummified lung tissue from an individual who died in Hungary in 1797 allowed genomic reconstruction of another *Mycobacterium* species, *Mycobacterium tuberculosis*, and suggested infection with two genotypes (13).

Turning back to brucellosis, Kay et al. (14) applied shotgun metagenomics to calcified nodules from the skeleton of a 14th-century male excavated from the medieval Sardinian settlement of Geridu. Although the authors had initially once again suspected tuberculosis, they instead were able to recover a *B. melitensis* genome. While coverage (6.5-fold) was somewhat low, the use of single nucleotide polymorphism (SNP) data in comparison with draft genomes emerging from an ongoing international *Brucella* whole-genome sequencing project sampling the global diversity of the genus (https://olive.broadinstitute.org/projects/brucella_ii, last accessed 18 July 2014) allowed phylogenetic placement of the genome. The genome was placed within a clade that includes the well-studied *B. melitensis* biovar 3 reference strain Ether, originally isolated from an Italian goat in 1961, two other more contemporary Italian isolates, and two additional isolates, one from Egypt and one of unknown origin. This placement was also confirmed by further analysis of deletions and IS*711* insertions, which were found to be confined to Geridu and the five other strains in the clade in a comparison with all other *B. melitensis* genomes available. In support of the work truly reflecting recovery of an ancient *Brucella* genome, sequence reads showed signatures typical of ancient DNA, and it was confirmed that work was performed in a laboratory with no history of work with *Brucella* cultures or DNA. It has been suggested that the initial success of shotgun metagenomic approaches applied to *Mycobacterium* might reflect the unique properties of the cell walls of these organisms in the form of a robust hydrophobic layer of mycolic acids that may protect mycobacterial DNA from damage (15). A particularly important aspect of this study is therefore the evidence that, under the right conditions, whole-genome sequences can be derived from organisms with more fragile cell envelopes at least hundreds of years postmortem.

Although these approaches are not without controversy (16), shotgun metagenomics applied to ancient material represents a powerful new tool with the potential to shed light on the emergence, evolution, and spread of microbial pathogens, both contemporaneously and stretching back into history and perhaps even prehistory. The technique has the potential to add new perspectives to our understanding of historical events where the causative agent may be unclear; in addition, it will be invaluable for understanding relationships between pathogens today and those in the past, providing clues to the causes of increases or decreases in virulence over time and possibly enabling calibration of the rate of evolutionary change. Specifically with regard to *Brucella*, if even older genomes could be recovered in the future from human and animal remains, these might add to our understanding of how *Brucella* evolved into the ecotypes seen today and shed light on the

historical and prehistoric associations between animal and human brucellosis.

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