ELSEVIER

Journal of Archaeological Science 31 (2004) 917–925

Journal of Archaeological SCIENCE

http://www.elsevier.com/locate/jas

从分子水平上检测出土的16世纪易洛魁族狗的整骨疗法在肺结核诱发增殖性关节炎上的应用

Molecular evidence of tuberculosis induced hypertrophic osteopathy in a 16th-century Iroquoian dog

——加拿大麦克马斯特大学

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Received 18 September 2003; received in revised form 28 November 2003; accepted 3 December 2003

实验中将动物腕骨经清洗后，置于CertiPrep公司生产的SPEX 6770/6870冷冻研磨机内研磨，所得粉末即用于DNA抽提

Abstract

A fully articulated dog skeleton excavated from a 16th-century Neutral Iroquoian site in Ontario, Canada displays a distinctive osteological condition known as hypertrophic osteopathy (HPO). Ancient DNA (aDNA) analysis of the dog has isolated *Mycobacterium tuberculosis* complex DNA, linking the secondary condition of HPO to tuberculosis (TB) and representing the oldest known case of TB yet to be discovered in the domestic dog. We emphasize that dogs should be considered as potential reservoirs of TB into the Americas.

Keywords: Ancient DNA; Dogs; Hypertrophic osteopathy; Iroquoian; Palaeopathology; Tuberculosis

1. Introduction

Ancient DNA analysis of the remains of a 16th-century Neutral Iroquoian dog excavated at the Cleveland site in southern Ontario, Canada has revealed the presence of *Mycobacterium tuberculosis* complex DNA, the causative agent of tuberculosis. This disease is transmitted through inhalation of infective airborne droplets, or through the digestive tract from oral contact with sputum, faeces or urine [28,48]. TB is anthropozoonotic, meaning that it is transmissible between humans and other species, including dogs [4,13,18,28,48]. Paleopathological and molecular studies have established the presence of pulmonary TB in human skeletal remains from the Americas (see [6,38]), but this is the first example of an archaeological dog confirming the condition. While TB is a recognized disease in modern dogs, instances are rare today due to the advent of effective chemotherapy and adequate veterinary care. The history of the condition in canids has yet to be established.

Iroquoian speakers inhabited south central Ontario from the Terminal Woodland Period ca. AD 1000 until European contact after AD 1550 [56]. The Neutral confederacy was part of the broader linguistic group designated as Northern Iroquoian [27,50]. The confederacy of Neutral tribes had been so named by the French colonists, as they appeared not to be at war with their Huron allies, the Iroquois, or amongst themselves [50]. William C. Noble excavated the Cleveland site (AhHb-7), a proto-historic Neutral village, in 1971. The site was inhabited ca. AD 1540+90 (Lab No. I-6514) [8,32,55]. The "Protohistoric" era refers to a period during which European trade goods preceded European colonizers in the region [27]. European artifacts found at the site secure its occupation to sometime after initial contact in AD 1500, but the infrequency of these goods at the site likely restricts its occupation to just prior to the advent of the commercial fur-trade in AD 1580 [27,32]. The Neutral confederacy was disbanded and displaced in the region by AD 1650 [50].

Iroquoian villages of the late Woodland period relied on horticultural subsistence economies, growing maize, beans, squash and other cultigens, as well as relying on wild resources such as terrestrial game and fish [27,39]. Villages were sedentary settlements, frequently encircled

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0305-4403/04/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jas.2003.12.006

by defensive palisades and consisting of several multi-family long houses that relocated every 10–30 years due to the depletion of the quality and availability of natural resources, such as fertile soil and firewood [51]. Populations were dense and aggregated, circumstances conducive to the accumulation of refuse and unhygienic conditions that promote the transmission of pathogens [40].

Dogs were kept within Iroquoian communities for a variety of purposes, including hunting and guardianship [15,25]. Ethnohistoric accounts document how the Iroquoians bonded with their dogs, sharing meal scraps, dwellings and even food from their mouths [49]. However, there was a duality to this relationship. In some instances, archaeological evidence also supports historical accounts of both sacred and secular dog consumption [42]. Dog remains from several late Iroquoian sites in the region, including Cleveland, produce evidence consistent with patterns of consumption, including disarticulation, cut-marks, breakage, burning, charring, gnawing and careless discard [3,39].

The dual role dogs held within the human community can be recognized in the archaeological record. While butchered and disarticulated dog remains were common components of the faunal assemblages from the Cleveland middens, discrete dog burials were also unearthed at the site [39]. In total, three burials were recovered from the middens, including two juveniles and the adult dog that is the subject of this study. The adult dog skeleton (C-10) was exceptionally well preserved and found fully articulated, "enmeshed" within a pot [8]. While human burials were usually relegated to interment inside a longhouse or outside the confines of the village in a local cemetery or ossuary, it seems dog remains were not subject to such mortuary restrictions. The ambiguous treatment of this burial, interred using customs usually accorded a human yet buried within the confines of the village with other dogs and exploited fauna, suggests a complicated relationship existed between humans and their dogs.

The Cleveland dog skeleton (C-10) was a mature male, based on the morphological characteristics of the skull and pelvis as outlined by Shigehara et al. [43], though no bacculum was recovered from the excavation. Dental wear was minimal and there was a lack of degenerative changes on the joints, indicating the animal was no more than 3 years of age at death [22,44]. Measurements of the fore and hind limbs based on formula by Crockford ([14], after Harcourt [20]) indicate he stood about 46.5 cm tall at the shoulder (Table 1), the average size of many Spitz-like breeds. Historical documents refer to a breed of indigenous dog that was common amongst the eastern Woodland populations [1,15,25]. This "common" dog was medium-sized and characteristically red, yellow, white or black in colour, with upright ears, a sharp snout and curled tail. The Iroquois raised and traded medium-sized dogs that were slight of build, with sharp muzzles [42]. A modern, registered breed called the Carolina Dog or American Dingo is believed to be a descendent of the indigenous "Indian" dog [31,42]. The main identifying characteristics include a straw through ginger-red colour, a 'fish-hook' tail, medium-size build, and upright ears. The standard average breed height of 43.2–61 cm makes it similar in size to the archaeological dog in this study.

Table 1
Estimated shoulder height of Cleveland dog

Element	Greatest length (mm)	Estimated shoulder height (mm) (after [14, p. 88])
Femur	151	461.18
Humerus	143	463.95
Tibia	154	459.09
Radius	144	477.43
Average shoulder height		465.27 (46.5 cm)

2. Skeletal evidence

2.1. Hypertrophic osteopathy

Hypertrophic osteopathy (HPO) has also been described in medical literature as hypertrophic pulmonary osteoarthropathy, hypertrophic osteoarthropathy, osteoporosis deformans, hyperplastic osteoperiostitis, tuberculosis osteopathy, and Marie's Disease, after the discoverer Pierre Marie [10,26]. Incongruities in the name reflect differences in the medical field's understanding of the pathophysiology of the disease, and earlier terms frequently refer to the condition as an arthropathy, a condition that would include joint involvement [26]. Today, it is recognized that the condition does not normally involve the joints and articular bone surfaces, so we prefer to use the term 'hypertrophic osteopathy', as a more accurate description of the disease (*Hypertrophic*—to grow abnormally, and *osteopathy*—disease of the bone). HPO is a progressive, symmetric and bilateral periosteal reaction secondary to chronic, space-occupying lesions of the inter-thoracic region such as pneumonia, pulmonary abscess, dirofilariasis, cancer or tuberculosis [2,10,21,28]. It is most commonly reported in dogs and humans, though other animals such as deer, cats, birds, and horses may also be afflicted with HPO [26,53]. The condition appears similarly in dogs and humans and is recognized by thick periosteal new bone exostoses, primarily on the appendicular skeleton. HPO is not transmissible, but the primary infection that causes the reaction may be. With treatment of the primary condition, the secondary reaction noticably regresses in 3–4 months [26].

The C-10 dog skeleton displayed proliferative periosteal new bone growth on the main elements of the

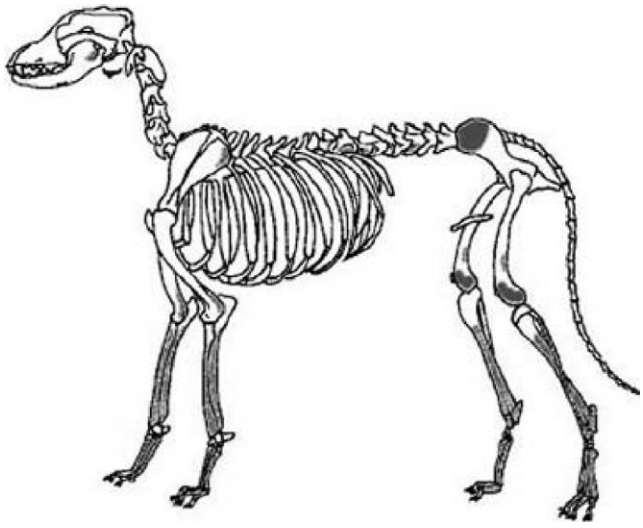

Fig. 1. Distribution of hypertrophic osteopathy (HPO) lesions on the Cleveland dog skeleton.

Fig. 2. Left (cranial aspect) and right (caudal aspect) radii with exostoses from hypertrophic osteopathy.

appendicular skeleton (Fig. 1). In 1973, the condition was diagnosed as hyperpulmonary osteoarthropathy by Dr Thomas Hulland, Dean of the Ontario Veterinary College at the University of Guelph, Ontario [8]. The only other known archaeological documentation of canine HPO in the Americas is from a pre-contact era, Late Woodland dog burial (ca. AD 500–1000) from a site in Alabama called Hickory Bend [11]. Though the condition is notably rare in modern dogs, without biomedical intervention there is little doubt that more cases would be reported. Sparse documentation of this condition in the archaeological literature may be a result of the relative rarity of fully articulated dog burials. The fragmented and disarticulated nature of most faunal assemblages would hinder recognition of this syndrome. If a single element that doesn't normally display lesions, such as the skull, was found, the presence of the condition could be entirely overlooked. This could lead to a failure to recognize and consequently, to report the condition.

The areas of proliferative periosteal growth on the dog skeleton are manifest most extensively on the weight-bearing regions of the body, the lower fore and hind limbs. The proliferation is symmetric, affecting both radii (Fig. 2), ulnae, tibiae (Fig. 3) and fibulae and is present on all metacarpals (Fig. 4) and metatarsals (Fig. 5). To a lesser degree, it appears on the distal portions of the humeri and femora, and it is active on some, but not all, phalanges. Minor periosteal reaction appears on the scapulae, os coxae and mandible. There is no visible reaction on the cranium.

2.2. Tuberculosis

In 3–5% of human cases (see [33] for review), pulmonary tuberculosis is manifest on the skeleton as a distinctive syndrome characterized by osteomyelitis in the weight-bearing regions of the body such as the hips, knees, and spine [34]. Three species of the *M. tuberculosis* complex are pathogenic in humans and other mammals: *M. bovis*, *M. tuberculosis* and *M. africanum* [28]. Microbiological studies indicate that dogs are most commonly afflicted by *M. tuberculosis* over *M. bovis*, though they are susceptible to both [45]. Differential diagnosis is difficult to establish from the skeletal lesions alone, as the disease can affect the bone in several ways, making any characteristic patterns difficult to identify. In carnivores in general, osteomyelitis with the development of draining fistulas, is common [28]. In dogs specifically, the skeletal involvement of TB results in bone cell proliferation rather than necrosis [45]. This pattern can be isolated in one bone or region, or involve major portions of the rest of the skeleton. In contrast, TB contributes to the demineralization of bone in humans, weakening the structure, which can result in bone collapse and fracture [52].

3. Molecular evidence

3.1. DNA extraction

Two complete carpals that did not display periosteal reaction were selected for molecular study. DNA extraction and amplification procedures were performed in

Fig. 3. Left (cranial aspect) and right (caudal aspect) tibiae with exostoses from hypertrophic osteopathy.

Fig. 4. Left metacarpals displaying proliferative periosteal reaction, characteristic of hypertrophic osteopathy.

Fig. 5. Left calcaneus and metatarsals displaying proliferative periosteal reaction, characteristic of hypertrophic osteopathy.

geographically separated laboratory facilities specifically designated for ancient DNA research. To decontaminate the samples, the carpals were placed in a 10% bleach solution for 20 min and rinsed several times with sterile water. Each sample was placed in fresh 1 N HCl for 1 min then transferred to 1 N NaOH for 1 min. They were rinsed several times with sterile water and placed under ultraviolet (UV) lights for 20 min. The samples were turned several times to expose multiple surfaces to UV irradiation. The treated carpals were ground using a SPEX liquid nitrogen grinding mill (ATS Scientific Inc., Oakville, ON, Canada). The combined weight of the bone powder from both carpals was approximately 600 mg, which was then incubated in 3 ml extraction buffer (0.5 M EDTA pH 8.0, 0.5% sodium dodecyl sulphate and 100 μg/ml proteinase K) at 60 °C overnight in a hybridization chamber [57]. The extraction solution was centrifuged for 20 min at 2000 *g* and the supernatant was passed through a Centricon™ microconcentrator (Millipore, Bedford, MA, USA). Before loading into a QIAquick™ PCR Purification kit spin column, 500 μl of

腕骨经处理后置于CertiPrep公司生产的SPEX冷冻研磨仪中研磨成粉末，再加入裂解液进行DNA抽提。样品经充分研磨后所得粉末均一性好，DNA产量得以保证。

PB Buffer and 15 μl of NaAc were added to the sample. The QIAquick™ PCR Purification kit protocol was followed with the addition of a 10-min incubation at 70 °C prior to the final centrifugation step. The sample was stored at −20 °C until assay. An extraction blank containing no bone powder was carried through the same procedure to ensure that the buffers were contaminant-free.

3.2. PCR amplification of aDNA

To avoid contamination, all PCR reactions were prepared in a HEPA filtered PCR containment hood using nuclease free UV irradiated ultrapure water and filtered pipette tips. Bone preparation and processing and PCR reaction set-up occurred in separate rooms with the inclusion of negative controls in each step. All negative controls were negative throughout the analysis.

The sample was assayed by PCR amplification of a 198 bp segment of the mitochondrial D-loop region to determine the presence of endogenous canine DNA. Primers CMT1: 5′-TCGAGGCATGGTGATTAAG and CMT2: 5′-ACCCCTACATTCATATATTGAATT were used in a PCR reaction mix containing 50 mM KCl and 10 mM Tris–HCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 1.5 mg/ml bovine serum albumin (BSA), 2.5 U AmpliTaq Gold (PE Biosystems, Foster City, CA, USA), 20 pmoles of each primer and 5 μl of DNA template. All PCR amplification was carried out using the GeneAmp™ Thermocycler Model 2400 (Perkin–Elmer Applied Biosystems) in a 50 μl reaction volume. PCR parameters were as follows: initial denaturation at 95 °C followed by 50 cycles of 94 °C for 30 s, 55 °C for 60 s and 72 °C for 60 s [23]. Canine mtDNA was amplified, confirming the presence of endogenous DNA.

The presence of tuberculosis was confirmed by three separate nested PCR reactions targeting the insertion sequence, IS *6110*, specific to the *M. tuberculosis* complex. The primers used for the initial amplification step were 5′-CCTGCGAGCGTAGGCGTCGG and 5′-CTCGTCCAGCGCCGCTTCGG as described by Eisenach et al. [17]. This 123 bp fragment of the repetitive 6110 insertion sequence (IS *6110*) has been previously amplified in a number of aDNA studies [5,19,30,35,47]. The use of previously published PCR amplification parameters made it possible to avoid using modern positive controls and to eliminate the possibility of cross contamination. Each 50 μl PCR reaction contained the above with the following modification: 3 mM $MgCl_2$. PCR parameters consisted of an initial denaturation for 12 min at 95 °C, followed by 40 cycles of 94 °C for 30 s, 66 °C for 60 s and 72 °C for 60 s.

The nested reaction was performed as described by Taylor et al. [47] using primers 5′-TTCGGACCAC CAGCACCTAA and 5′-TCGGTGACAAAGGCCAC GTA to amplify a 92 bp product. Each 50 μl PCR reaction contained the above with 3 μl of the previous reaction being substituted for the DNA template. PCR parameters consisted of an initial denaturation for 12 min at 95 °C, followed by 30 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. The PCR products were run on 8% acrylamide gels using 1 × TBE electrophoresis buffer and visualized in an UV gel documentation system after staining with ethidium bromide. All samples were purified using the QIAquick™ PCR Purification kit before sequence analysis.

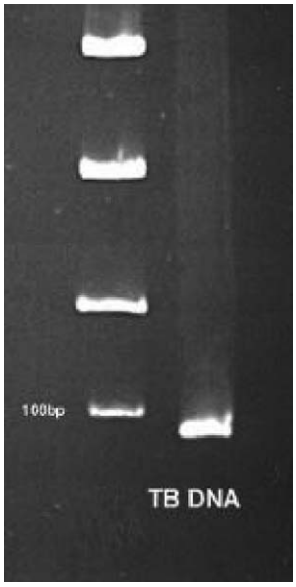

Fig. 6. *M. tuberculosis* complex DNA isolated from Cleveland dog skeleton.

Cycle sequencing of PCR products was performed using the ABI Dye Terminator Ready Reaction Kit (Perkin–Elmer Applied Biosystems) according to the manufacturer's protocol and analyzed on an ABI 3100 Genetic Analyzer. Sequence alignment was performed using the BioEdit computer software package. The 92 bp fragment of IS *6110* amplified and sequenced from the C-10 dog skeleton (Fig. 6) confirms the presence of *M. tuberculosis* complex DNA, implicating the bacterium as the causative factor in this case of canine HPO.

4. Discussion

4.1. Opportunities for transmission

The opportunity for transmission of tuberculosis was enhanced by living practices within the Neutral community. The direct consumption of dogs was not the only means of disease transmission, though disarticulated, broken and burnt dog bone recovered from the Cleveland site middens does attest to this dietary practice [3,39]. The Neutral disposed of household wastes in middens that were located within the confines of the

village. The scavenging and coprophagic habits of dogs would also have brought them into regular contact with the middens, salvaging what scraps they could and aiding in the transmission cycle of infectious pathogens. The *Jesuit Relations* are an ethnohistoric compilation of primary accounts that document European and Native relations during the 17th-century contact era among the Iroquoians. Between AD 1639 and 1640, it was recorded that dogs at that time were common and often numerous in villages, "… being held as dear as the children of the house, [sharing] the beds, plates and food of their masters" [49]. This sharing of food and eating vessels with dogs would have brought the two species and their respective pathogens into regular, and potentially transmissible, contact.

The burial of the C-10 dog also illustrates an opportunity for transmission by demonstrating the likelihood of human care for this particular animal. With HPO, the dog would have presented with swollen limbs. Movement would have been avoided and difficult on those limbs that were hard, taut and warm to the touch [21,26]. If the TB infection was also active, it is likely that the dog experienced laboured breathing and a productive cough, and may have appeared emaciated [45]. The advanced condition of the skeletal symptoms combined with the treatment of the dog after death suggests a nurturing relationship between this animal and its caregiver(s). Not only was this animal nursed to an advanced stage of illness, it was interred with the sort of care and respect usually allotted to another human. The lack of any evidence to suggest burning, cutting, or disarticulation highlights the fact that this dog was not consumed after death as many other dogs in the village had been. However, the type of close relationship suggested by the archaeological evidence would also provide the conditions necessary for the transmission of a primary TB infection between species. Although the path of original transmission cannot be discerned, there is a strong indication that the presence of the TB bacterium in one species signals its presence in the other.

Skeletal evidence of tuberculosis has been documented in Iroquois ossuaries, confirming the presence of the bacterium in indigenous human populations prior to European contact [5,36,40]. Ossuary burial was the predominant mortuary practice among the Iroquoian speakers of the region prior to contact in the mid 16th century [37,46]. In an ossuary-style burial, the individual remains were defleshed, disarticulated and reburied in a communal location outside of the main village. This type of communal burial practice provides a number of limitations for archaeologists and physical anthropologists due to the often brittle and disarticulated nature of the assemblage, which can make the diagnosis of pathological conditions difficult. There is also no independent means of identifying the members of an ossuary with a specific village, thus limiting the ability to link evidence of tuberculosis with the epidemiological context of a founding settlement.

In contrast, the dog burial was inhumed within the confines of a specific village. As the animal was buried fully articulated, the advanced nature of the skeletal syndrome was readily apparent. The uniqueness of this burial garnered an unusual amount of attention from excavators and analysts, making possible the identification of the definitive presence of TB within the context of a single, sedentary, inhabited location. As it was found in situ, it is likely to be an accurate reflection of the health conditions in this specific village. Although we cannot state with certainty that this dog resided its whole life at this site, its careful interment within a household midden strengthens this likelihood. It is possible, however, to conclusively state that TB was present at this Neutral Iroquois village some time during the period AD 1450 to 1630, a point that cannot be deduced from human ossuary burials alone.

The morphological identification of HPO in ancient dog or human populations provides a non-specific indicator of an underlying systemic disease. The pathogenesis of HPO is still poorly understood, however, tuberculosis has frequently been implicated as a common primary cause of the condition in the medical literature [10,21,26]. A recent palaeopathological and molecular study by Mays and Taylor [29] has also linked hyperpulmonary osteopathy with tuberculosis infection in two mediaeval human skeletons from Wharram Percy, England. Thus, while HPO is a secondary complication of a primary disease and TB is not necessarily the cause, finding even a few scattered or disarticulated remains within an archaeological collection that exhibit this condition is enough to now warrant further molecular testing in order to identify the primary pathogen.

As an anthropozoonotic infection, tuberculosis may be prevalent in both hunter-gatherer/pastoralist groups that have contact with animals in their environment, and in more urbanized settings where human to human transmission of pathogens is more likely to occur. As a highly contagious pathogen with a moderate morbidity rate and a relatively low mortality rate, *M. tuberculosis* can be maintained within even small population pools [40]. Since pathogenic bacilli can be harboured without any signs of active infection or skeletal lesions, the antiquity of the disease and its entry into the Americas prior to European contact is difficult to trace. Current research on this issue (for overviews, see [5,6,34,38]) should consider the dog as a possible reservoir or vector of TB into the Americas. Of relevance today, the incidence of TB in modern dog populations has been monitored at a rate of 1–5%, [28] highlighting the need for current TB surveillance programs to also consider dogs as an etiological reservoir of human infection.

4.2. Future research

In archaeological studies, dogs have been compared to assess their representativeness as human proxies in palaeopathological studies [3] and used as human surrogates in dietary isotopic studies [7,9,12,16,24,41,54]. The short life span of a dog in relation to humans allows us to investigate shorter periods of archaeological time, and since dog bone remodels much more rapidly than human bone, changes in reaction to stressors may be more immediately reflected in dog remains. The presence of palaeopathological lesions in response to pathogen load may develop more quickly in dog skeletons and act as indicators of overall stress in the human population despite the lack of evident pathology on human remains that might take much longer to materialize. As a result, anthropozoonotic diseases such as periodontal disease, Chagas' disease, ascariasis, Paget's disease, and of course, tuberculosis, are obvious candidates for investigation in archaeological canine assemblages through morphological, chemical and molecular methods.

The origin and development of HPO is still poorly understood, and Lenehan and Fetter [26] suggest that dogs would make excellent surrogates for humans in further studies of the pathogenesis of the condition. Archaeological collections add a temporal dimension to epidemiological studies and provide a sample base that was not subject to medical intervention. This canine example complements Mays and Taylor's [29] study of human remains, that demonstrated how molecular analysis helped to identify the primary pathogen that stimulated secondary HPO in their skeletal sample. The process of the infection, its incidence in a given population, and the primary etiology may all be better understood by investigating the archaeological context of dog remains afflicted with HPO. Multiple lines of evidence can also help to distinguish a number of epidemiological factors that may have contributed to HPO in the past, including the subsistence base of those infected, the population size at risk and any ecological conditions such as climate or seasonality.

5. Conclusions

The recognition of HPO in canine skeletal remains provides ancient health researchers with a non-specific but morphologically recognizable indicator of a primary disease process that is most likely pulmonary in nature. Molecular analysis can aid in diagnosing the primary infection, as systemic pathogens such as tuberculosis leave genetic signatures in bone, even when morphological evidence is absent. While human skeletal remains can offer a wealth of demographic and pathological information about past populations, such material is often difficult to access. Assessment of the impact of disease on archaeological human populations using molecular methods is therefore limited. Dogs, as our oldest domestic companions, are frequent components of archaeological assemblages the world over. These archaeological populations offer the added benefit of time depth to epidemiological studies of disease. Used in tandem, osteological and molecular analyses of dog remains present new and exciting opportunities for ancient health and disease research.

Acknowledgements

We wish to thank Shelley Saunders for the use of the laboratory facilities at the McMaster Palaeogenetics Institute, and Dennis Ng for his technical expertise. We are also grateful to Aubrey Cannon, Ann Herring and Patrick Horne for their helpful comments on earlier versions of this paper.

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