

Identification of Historical Human Skeletal Remains: A Case Study Using Skeletal and Dental Age, History and DNA

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ABSTRACT Excavation of an early historical (circa 1900) church cemetery in Cochrane, Alberta, Canada, revealed the graves of six individuals. All but one had been previously excavated and the individuals were re-interred elsewhere. The remaining grave contained a coffin burial of an infant including most of the skeleton, crowns of forming deciduous teeth, scalp and hair. The remains were excavated and historical research was carried out to determine possible families who had used the cemetery. Skeletal and dental age estimates pointed to a particular individual described in historical records. This identification was confirmed through more precise ageing by dental microstructure, sex determination using DNA, and finally, maternal relatedness by comparison of mtDNA with a living female relative. In addition to describing the analytical methods used, this paper demonstrates the importance of accurately identifying historical burials, particularly when living relatives remain in the community. Copyright © 2004 John Wiley & Sons, Ltd.

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Historical cemeteries are often encountered in the course of modern urban expansion. Physical anthropologists have been involved in studies of human remains from historical cemeteries in order to aid in identification and to learn more about the lives of people from the past (see, for example, chapters in edited volumes by Saunders & Herring, 1995; Grauer, 1995). Unmarked cemeteries may be encountered by accident or their presence may be known in advance. In the

former situation, it is first necessary to determine the identity of the cemetery population. In the latter case, the population affinity is usually known, but it may be desirable or necessary to determine the identity of certain individuals. This is particularly desirable when members of the living community wish to rebury the remains. In North America, considerable attention has been paid to excavations of native American and African American cemeteries and the accompanying need to involve those communities (e.g. Roberts & McCarthy, 1995). Local communities of European ancestry are also interested and wish to be involved in the removal and study of historical remains (e.g. Saunders *et al.*, 1995).

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Standard methods in human osteology provide a starting point for such identification; however, individual identification requires more sophisticated methods, including age determination by dental microstructure (reviewed by FitzGerald & Rose, 2000) and analysis of mitochondrial and nuclear DNA (reviewed by Kaestle & Horsburgh, 2002). These methods, in combination with research on historical documents, can result in positive identification.

Here we report on such a case where a known burial ground was excavated and positive identification was achieved through a combination of scientific and historical research. While this particular study reports on only six graves and just one individual, the methods described can be applied to other historical cemeteries where there is a desire to identify some or all of the individuals. The results can be used to better understand past health and lifestyle (e.g. Larsen *et al.*, 1995), and are also immensely interesting to individuals in the local community.

Background

In the summer of 1998, a small early historical church cemetery in Cochrane, Alberta, was excavated in order to allow for expansion of the town library. Cochrane is located 38 km (24 miles) west of Calgary. It began as a ranching community in the late 19th and early 20th centuries and historical records document the families that lived in the area. The town of Cochrane requested the excavation and removal of several historical graves that had been discovered accidentally during previous building activity in 1984. A small Catholic church had occupied the site since 1895 and the small cemetery associated with the church was in use from 1895 to 1905. During archaeological excavations six graves were exposed, but it soon became apparent that, with one exception, all of the individuals had been moved to other cemeteries, probably prior to 1910. One coffin remained and contained the remains of an infant. Because many of the early settlers have descendants in the area, it was important to establish the identity of the individual so that the place of reburial could be decided (that is, within a particular family plot or

as an unknown pioneer in the town cemetery). Such investigations are of interest to the local community and provide an opportunity to communicate the methods of recovery and identification using sophisticated scientific methods.

The excavation

Following the discovery of the cemetery in 1984, a small monument was placed over the area and a tree was planted. In 1998, following removal of the monument and tree, ground-penetrating radar was used to determine the number and location of graves. Four graves were located in this manner and two additional graves were found under utility pipes as a backhoe removed the surface layers of sod and soil. Each shaft was carefully excavated, but only one contained human remains. In two shafts, parts of the original coffins were discovered and another contained the remains of a wreath. At the east end of three of the graves, the remains of wooden posts, presumably crosses, were found. Thus the graves were marked at the time of burial, but no record of who was buried there remained. A row of posts approximately one pace from the east end of the graves indicated the former presence of a picket fence. Collectively this evidence indicated that there had been a small cemetery associated with the former St. Mary's Church.

The dimensions of the graves, and, by inference, the size of the coffins, was used by the archaeologist (GO) and the historian (JO) to reconstruct the stature and possible age of individuals. Size of grave shafts varied and indicated that two adults and four children had been buried there. The arrangement of the graves relative to the church and the fence, along with historical artefacts (nails and coffin hardware) included in the grave fill, provided information on the dates and sequence of interment. The historian on the project, Joy Oetelaar, worked with archival records, newspaper stories and the local church to determine who was likely to be buried in the cemetery. The small church was constructed in 1895 and a new community (non-denominational) cemetery was established on higher ground in 1905. At that time, individuals buried next to the small church in Cochrane would have

Table 1. Skeletal and dental age estimates

	Measurement (mm)	Estimated age (months)	Reference
Deciduous crown formation		2–4	Moorrees <i>et al.</i> (1963)
Diaphyseal length (tibia)	82	2–4	Fazekas & Kosa (1978); Saunders <i>et al.</i> (1993a)
Length (ilium)	43	6–12	Saunders <i>et al.</i> (1993a)
Length (occipital bone, basilar portion)	15	Late fetus—3	Redfield (1970)
Breadth (occipital bone, basilar portion)	14.5	Late fetus—3	Redfield (1970)

been moved either to the new Cochrane cemetery, or to another cemetery of the living family's choice. A list of all those who died between 1895 and 1905 was compiled along with information about their ages at death. The archaeological and historical evidence led to the identification of specific families and to individuals from those families who had died during the time the cemetery was in use. This ultimately led to the conclusion that the infant was related to individuals currently living in the community. Their cooperation in the mitochondrial DNA analysis led to the identification of the infant.

The smallest shaft is the one that still contained a coffin and an individual within. As the top planks of the coffin were removed it was apparent that hair and scalp as well as bones were preserved in the infant burial. A bow and fragments of a cloth coffin liner were also present. However, the bones of the infracranial skeleton were poorly preserved. The differential preservation is attributed to the fact that the head rested on a dense mat of wood shavings—probably the stuffing of a pillow. Tannins in the wood acted as a soft tissue preservative. However, the coffin collected moisture such that bone preservation was very poor. Only two bones were complete enough to obtain measurements for the purpose of assessing age at death (left ilium and right tibia). Crowns of nine developing deciduous teeth were recovered and these provided the most reliable age indicators.

Methods and results

Age determination

Deciduous tooth crown formation indicates an age of 3 months \pm 1 month (Moorrees *et al.*,

1963). Measurement of two intact bones supports the estimate from deciduous crown development. The diaphyseal length of the right tibia is 82 mm and the breadth of the left ilium is 43 mm. The tibia length corresponds to an estimated body length of 64.3 cm (Fazekas & Kosa, 1978). This body length corresponds to an age estimate of 2 to 4 months, based on a comparison with measurements from a 19th century Anglican cemetery in Belleville, Ontario (Saunders *et al.*, 1993a). Finally, the length and breadth of the basilar portion of the occipital bone is consistent with that of a young infant (Table 1) (Redfield, 1970). Some of the right and left halves of the neural arches of thoracic and lumbar vertebrae were fused. Specifically, five thoracic and three lumbar vertebrae exhibited fusion of the right and left halves of the neural arches. This normally happens toward the end of the first year of life (Scheuer & Black, 2000) and is therefore inconsistent with the other age indicators. Problems with dental age estimates in infants have been detailed by Saunders *et al.* (1993b) and by Liversidge (1994).

Another age estimate came from the historical research. A stone monument in St. Mary's Cemetery in Calgary lists family members of individuals identified in the historical documents, along with birth and death dates (1860s to 1904). At the bottom of this monument is an inscription made prior to 1920 with a female name (MEM) and lifespan ('Aged 5 months') but no dates. Because the skeletal and dental data provide an age range of two to four months and because the historian discovered this possible identification of a female infant who died at the age of five months, we sought to obtain a more precise age estimate through enamel microstructure analysis. If that analysis placed the age at death toward the upper end of the range, we then

wanted to determine the sex of the individual using DNA. Three of us (MAK, GO and JO) made a presentation to the town on our findings and our staged plans to work toward a positive identification. At that time we received permission from the community and from the possible descendants to carry out two types of destructive analyses.

Firstly, three deciduous tooth crowns were selected for age determination by enamel microstructure. Upon receipt of those results, samples from both scalp and bone were analysed for DNA sex estimation. Hair samples from three of the people who had most closely handled the infant during and after excavation were also analysed. Following the initial results, further hair samples from a living relative (the baby's niece) were tested for mtDNA.

Microstructural analysis of the deciduous teeth

This method is based on the interpretation of histological markers in tooth enamel and dentine that record growth, thereby providing an endogenous record of development. In contrast to all other methods of age-at-death estimation, this approach removes the need to apply standards of any sort, allowing accurate assessments based on calibrations internal to the tooth itself. There are other histological methods for determining age from teeth, such as counting layers in cementum (Stott *et al.*, 1982; Miller *et al.*, 1988), Gustafson's method of multiple determination (Gustafson, 1950; Burns & Maples, 1976; Lucy *et al.*, 1994; Lucy & Pollard, 1995), and dentine sclerosis (Bang & Ramm, 1970; Johanson, 1971; Lucy *et al.*, 1994), but these all apply to adults. In fact, a limitation of the dental microstructure technique is that it can only be used to determine ages in individuals who have not reached dental maturity and who still have at least one tooth that has not completed its growth.

Papers by FitzGerald (1998) and FitzGerald & Rose (2000) include detailed surveys and descriptions of previous research and provide a solid rationale and evidence for dental microstructure ageing. Here we offer a short description of enamel development in order to make the method understandable. Although it is possible to esti-

mate the time taken for root growth from dentine, the precision of this technically challenging method is not sufficient for the Cochrane case.

Tooth enamel is formed from hundreds of thousands of unbroken, interlaced prisms that extend from the enamel–dentine junction (EDJ) to the tooth surface (Plate 1). Prisms are produced by ameloblasts that secrete enamel matrix from their distal ends as they slowly make their way to the surface of the tooth. Very shortly after its secretion, the matrix begins to mineralise and mature into enamel. When the ameloblasts reach the tooth surface, they lose their columnar shape and are eventually shed during tooth eruption. Therefore, there is no possibility for subsequent enamel repair once the tooth crown is formed.

Microstructural growth markers within the enamel are grouped into two basic categories: short period markers and long period markers. Short period markers, also called *cross striations*, result from the metabolic changes arising through one of the body's central regulators, the circadian or 24-hour rhythm. Under polarised light, they appear as bands consisting of fine, transverse, dark striations along the length of enamel prisms (Plate 1). Cross striations result from variations in the degree of mineralisation of enamel matrix as well as the rate of secretion; matrix is secreted faster at times of most intense metabolic activity (Boyde, 1979, 1989). Mineral density varies as a result of variations in the ratio of calcium and phosphate to magnesium and carbonate in the hydroxyapatite of enamel crystallites, a shift toward the latter being associated with a reduction in density.

The long period markers of enamel are also called *brown striae of Retzius*. In longitudinal sections (i.e. in two dimensions), brown striae of Retzius appear to form successive layers (or 'caps') around the dentine horn. After the first cap reaches the occlusal surface, each subsequent layer does not fully cover the previous one, but partially overlaps it at the surface. Striae layers continue down on either side of the crown all the way to the cervix or neck of the tooth, most striae appearing to run obliquely from the EDJ to the occlusal surface so that they form an acute angle with the prisms they cross. Striae differ in

their visibility and they are variably expressed, even within one tooth. They are most clearly discernable in the outer enamel, and particularly also in the cervical third of crowns.

The number of daily increments (cross striations) between adjacent long period lines in enamel is uniform within one tooth and is consistent among all of the teeth in one dentition (FitzGerald, 1998). However, the number of cross striations between adjacent striae, called a *circaseptan interval* (because it is 'around seven [days]'), although uniform in all teeth of one individual, commonly varies from 7–11 days among different individuals. It has been suggested that this near-weekly rhythm might arise from interference beats between several interacting rhythms (Newman & Poole, 1974, 1993).

The technique for calculating age-at-death in permanent teeth usually relies on the uniform periodicity of circaseptan intervals, which are used to determine the amount of enamel formation in days from birth. However, because microstructures are not as clearly expressed in deciduous teeth, long period markers cannot be used. Nor is it possible to simply count the total number of all cross striations (i.e. days elapsed) since cross striations are rarely discernable through the full depth of the enamel mantle in deciduous teeth. Therefore, the rate of enamel growth per day must be determined from points where cross striations are easily seen and interpolated through prism length from birth. Fortunately, the birth event is readily identifiable from the presence of one particularly accentuated stria of Retzius, the neonatal line (Plate 1). Neonatal lines are hypothesised to arise from physiological changes occurring during the birth process (Sognaes, 1949; Gustafson & Gustafson, 1967; Weber & Eisenmann, 1971; Whittaker & Richard, 1978; Whittaker & MacDonald, 1989; Eli *et al.*, 1989; Skinner & Dupras, 1993). While it is not entirely clear how much time it takes to form the neonatal line, there is indirect evidence from total cross striation counts of the crowns of personally identified individuals that the period is closely localised to the birth event (Antoine *et al.*, 1999; Antoine, 2001).

Consequently, the method proceeds as follows (Plate 1):

- (1) Identify the neonatal line in a tooth section, which then sets to 'zero' the chronology of enamel formation.
- (2) Determine the average cross striation repeat interval (representing one day's growth of enamel) along a prism running from the neonatal line (A) to the point where growth has ceased at the enamel surface (B).
- (3) Measure the length of this prism.
- (4) Determine the age in days by dividing the length of the prism by the average growth rate per day.

Results of microstructural analysis of the deciduous teeth

The three deciduous teeth that were analysed were the maxillary lateral incisor, the canine, and the maxillary first molar. Each tooth was embedded in Buehler Epo-Kwick[®] epoxy resin and then sectioned. Two sections were taken from each tooth, a longitudinal cut through the centre and a second cut, taken in case the centre section suffered damage, offset from the centre by approximately 200 µm.

Maxillary i2

Unfortunately, some damage had occurred slightly above the point of contact of the neonatal line with the EDJ in the centre thin section of this tooth. Because of this damage the calculated postnatal time of crown development, 2.1 months, is considered to be an underestimate. The estimate of crown formation for the offset cut was calculated at 2.4 months. However, the infant lived beyond this age because the root of the tooth had already begun to form, although it was in its initial stages of growth. While the determination of 2.4 months for full crown completion for this tooth is in agreement with expectations from morphological studies in the literature (Lunt & Law, 1974), this result cannot be used to determine age at death.

Canine

It was only possible to make one section through this tooth germ, which was tiny and extremely fragile. The microstructural clarity in the enamel was good, but the enamel had broken away at a point before the intersection of the neonatal line with the EDJ so that it was impossible to

determine the full time of crown development. An estimated age of 4.1 postnatal months derives from the enamel that remained undamaged. This should be viewed as an underestimate.

Maxillary m1

Two sections were prepared from this tooth. The buccal side of the enamel was cracked and broken away before the intersection of the neonatal line with the EDJ, therefore the lingual side was used to estimate age at death. Some slight damage to the enamel surface was present, but this had no significant effect on determining age since cross-striation counts were visible, as were the total number of long period markers or striae of Retzius. The postnatal time taken to grow the crown before death, taken from the centre cut thin section of this tooth, is 4.8 months.

Conclusions from microstructural analysis of the deciduous teeth

The lateral incisor was crown complete at about 2.4 months of postnatal age. This means the result cannot be used to determine age at death, except to specify the lowest bound. However, there is a consistency in the results obtained from the other two teeth. The best estimate for this infant's age at death was determined from the centre section of the maxillary m1 to be 4.8 months. The enamel of the canine tooth was too damaged to indicate a full age at death, although the calculated minimal estimate of 4.1 months is consistent with the putative age at death determined from the maxillary m1. Age at death estimated from the offset section from the maxillary m1 is 5.1 months. Because age was estimated by determining the rate of growth from two or three points (depending on the tooth and on the section) where cross striations are discernable and interpolated through prism length rather than counting all days to grow a prism, it must be assumed that there is some level of inaccuracy. The 'true' chronological age probably lies within the range of 4.8 to 5.1 postnatal months.

DNA analysis

Initially, samples of scalp and hair as well as bone were sent to DY to extract DNA for sex

estimation. Unfortunately, both male and female results were obtained from the first set of samples, which would indicate contamination. However, a unique mtDNA sequence was observed from almost all DNA extracts, indicating positive preservation of DNA from the samples. Consequently, a second set of samples containing larger amounts of tissue were sent some months afterward and these were tested separately.

Sample preparation

To reduce chances of contamination, approximately 0.5 gram of bone and soft tissue from the second set of samples were used for each extraction and four separate DNA extractions were carried out for each sample. Chemical decontamination methods were used to destroy possible surface contaminant DNA, including the use of bleach, HCl and ultraviolet light to maximise the effectiveness of decontamination, even though this approach might also damage endogenous DNA. All extractions were carried out in a dedicated laboratory using all appropriate procedures to prevent contamination, including protective gowns, masks, caps and gloves as well as the cleaning of counters, equipment and instruments with bleach and ultraviolet light.

A liquid nitrogen-grinding mill was used to generate fine bone powders for the extractions. The silica-spin column method (Yang *et al.*, 1998a) was used to extract DNA from the bone samples. The bone powders were incubated overnight with lysis buffer (0.5 M EDTA pH 8.0, 0.5% SDS and 0.5 mg/mL proteinase K) in a rotating hybridisation oven at 55°C. After centrifugation, 1.5–2.0 ml of supernatant was concentrated using Centricon™ 30 microconcentrators (Amicon Division, Danvers, MA). Approximately 100–150 µl of concentrated supernatant was passed through QIAquick columns (Yang *et al.*, 1998a). Finally, 100 µl of DNA was eluted from the column for PCR amplification.

For this identification, hair root samples from three of the people who had most closely handled the infant during and after excavation were also submitted. Finally, a hair sample was obtained from an individual believed to be a living relative of the Cochrane infant. The

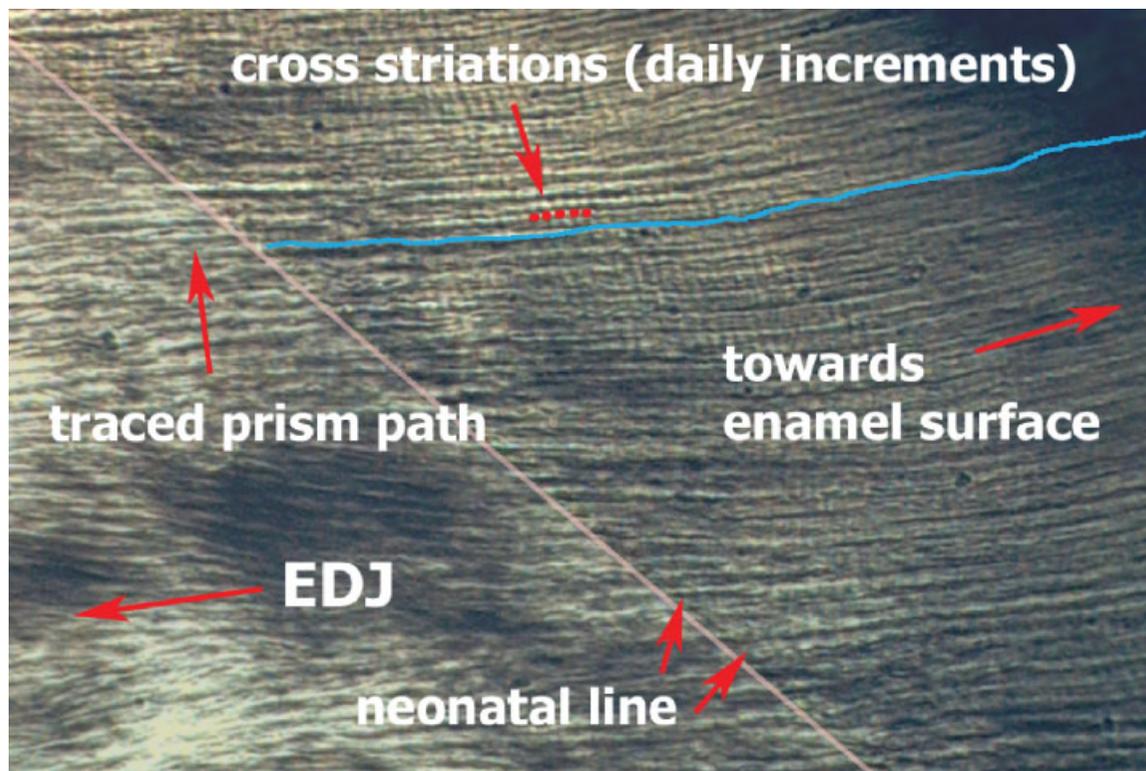


Plate 1. This is a thin section of a portion of the deciduous molar analysed in this study, photographed at $400\times$ magnification. Prisms can be seen running in an approximately horizontal direction from the left (originating at the enamel dentine junction, which is out of frame in this photomicrograph) towards the cuspal surface on the right (again, out of frame). Cross striations, representing daily increments of growth, can be clearly seen in the upper central portion of this photograph (five have been highlighted with red dots). In areas like this, average daily growth rates can be easily calculated by measuring the distance between sequential cross striations and taking a mean. The neonatal line is difficult to see in this image, and a pink line has been drawn just to the left of it to aid identification. It runs in an approximate diagonal from bottom centre-right to upper left. The prism path used to estimate age at death in this tooth has been over-scored in blue and, as explained in the text, age is estimated by first measuring the distance along this prism from the tooth surface to the neonatal line and then dividing this figure by the daily rates of growth established along its length.

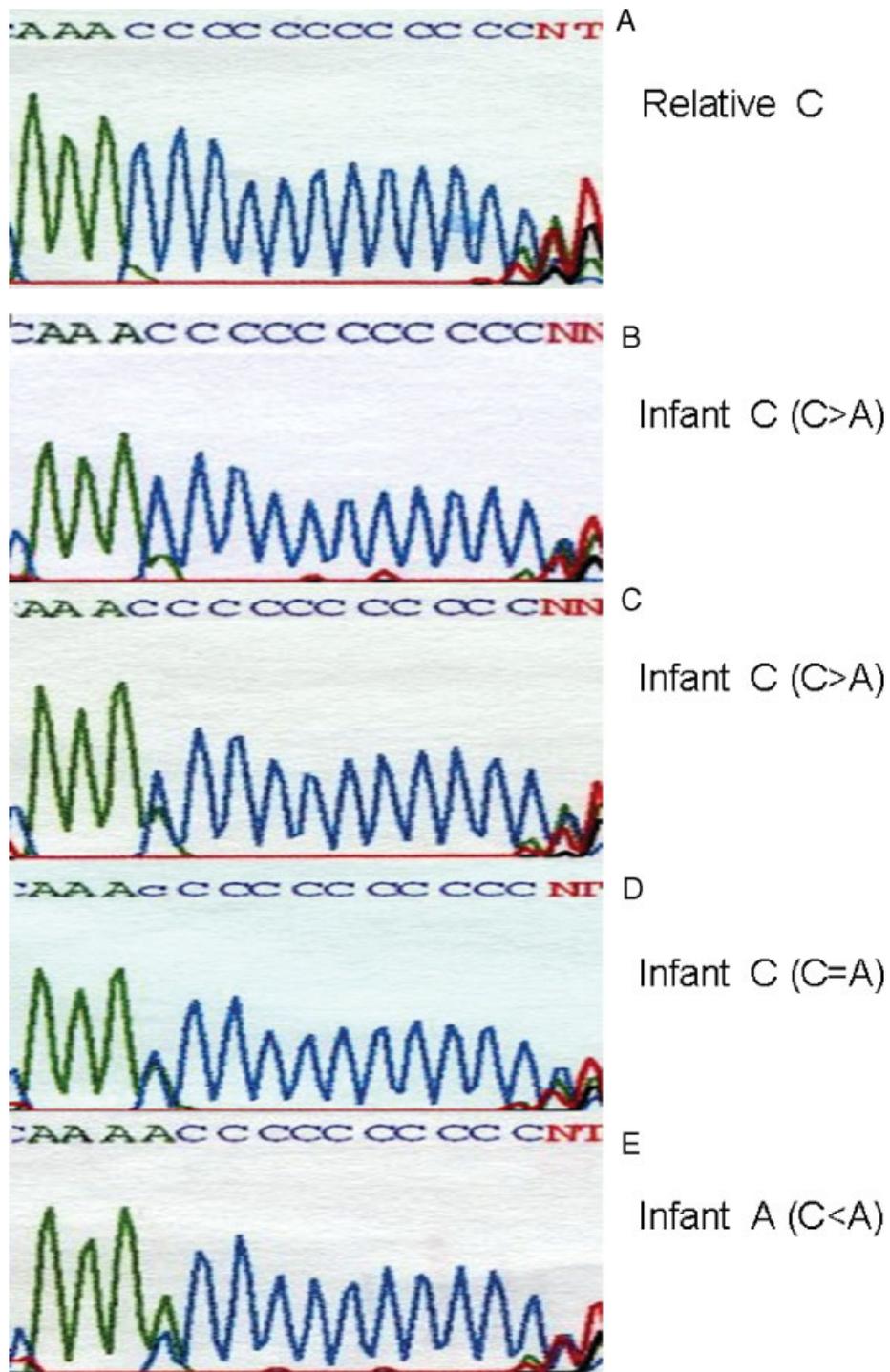


Plate 2. Sequencing electropherograms to show np 16,183 for the relative and the infant samples. For the relative, A: a clear C*; for the infant, B and C—a predominant C; D—seemingly equal C and A; E—a predominant A. (*The trace in green is most likely a sequencing artefact since a similar pattern in green was also seen across this electropherogram.)

hypothesis was established to test for a genetic relationship between the infant, judged to be individual MEM from historical research, and the living daughter (BF) of MEM's presumed sister.

In this study, we amplified the first and second hypervariable segments (HV1 and HV2) of the mtDNA control region (Anderson *et al.*, 1981) in overlapping fragments in a similar manner to the methods of Stone & Stoneking (1996, 1998). Primers for the smaller fragments, each less than 300 base pairs, were designated as fragments mt1 to mt4 (see Figure 1). Three sets of primers (mt1-1/mt1-2, mt2-1/mt2-2 and mt3-1/mt3-2) were used to amplify the HV1 region and the first half of the HV2 region of the D-loop area of human mtDNA, and one set (mt1-1 and mt4-2) for the entire D-loop for modern DNA to test the presumed living relatives and three archaeologists who handled the remains. PCR amplification was carried out using a GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk, CT) in a 50 µl reaction volume containing 50 mM KCl and 10 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 0.2 mM dNTP, 1.0 mg/ml BSA, 0.3 µM each primer, 5 µl archaeological DNA sample and 1.25 U AmpliTaq Gold™ polymerase. PCR was run at 35–38 cycles at 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds with an initial denaturing at 95°C for 12 minutes. Five µl of PCR product was separated by electrophoresis onto an 8% non-denaturing polyacrylamide gel. PCR products were purified using a QIAquick™ purification kit. The samples were sequenced on an ABI 373 at the Central Facility of the Institute for Molecular Biology and Biotechnology, McMaster University. Because a C instead of a T presented at HV1 position 16189, blurred sequencing electropherograms were encountered for both the infant and relative's

samples, so that sequencing from the other strand was carried out to obtain the complete sequencing result.

Mitochondrial DNA analysis

The high copy number of mitochondrial DNA, several hundred molecules per cell (Robin & Wong, 1988), improves the chances for success when analysing mtDNA as opposed to nuclear DNA from historical samples. In addition, the rapid rate of evolution of mtDNA increases the probability of exclusion if the historical remains are not from the individual in question. Finally, in contrast to nuclear DNA, which undergoes segregation and recombination, the haploid and maternal inheritance of mtDNA means that any living maternal relative should have the same mtDNA type to the individual or individuals being investigated. Several published examples serve to illustrate the success of this approach (Gill *et al.*, 1994, 1995; Ivanov *et al.*, 1996; Stone *et al.*, 2001).

Most of the DNA extracts from the Cochrane tissue samples produced good amplifications of mtDNA, although some fragments were more easily amplified than others depending upon primers and individual extracts. Multiple amplifications were run from the different extracts from both the first and second sets of samples taken from the Cochrane case. The mt1 fragment was sequenced 12 times, the mt2 fragment three times and the mt3 fragment six times. The reconstructed mtDNA sequence for the HV1 region comprises a total of 371 nucleotides as well as 259 nucleotides for the first part of the HV2 region. The sample from the proposed living relative was also sequenced two to three times depending upon the fragment. Tissue

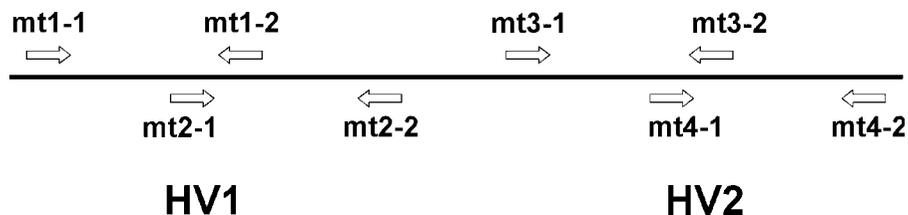


Figure 1. PCR amplifications of small DNA fragments.

samples from each of the three individuals who had handled the remains were amplified and sequenced once for all fragments.

For the HV1 region, the Cochrane individual has four base pair differences (16183 A/C, 16189 T/C, 16207 A/G and 16356 T/C) when compared with the published reference sequence. Mitochondrial DNA analysis, as with any DNA test, is a test for exclusion. If the mtDNA sequence of the case sample is different from the maternal source sample, then the probability of exclusion is 100% (barring sample mix-ups or laboratory errors).¹ However, the same sequence was obtained from the proposed living relative as was found for the Cochrane infant, leading to two possible interpretations: that the exhumed remains are indeed the remains of a maternal relative of the living individual BF who was tested; or the remains are from an unrelated individual who, by chance, happens to have the same DNA sequence as the archaeological sample. To examine the probability of detecting an unrelated individual we searched the HVRbase database of approximately 5000 mitochondrial HV1 sequences sampled from living (and some ancient) individuals (Handt *et al.*, 1998). There were no cases of individuals who shared the same group of nucleotide differences as for the Cochrane case and individual BF.

The differences at sites 16189, 16207 and 16356 are all transitions, while the difference at site 16183 is a transversion from A to C. A review of the literature shows that the nucleotide change from A to C at this position is not uncommon and forms part of a group of hypervariable sites that are usually assumed to represent mutational hotspots (Meyer *et al.*, 1999; Stoneking, 2000). Much earlier, Horai & Hayasaka (1990) identified a 20 base block ranging from 16178 to 16197 as a hypervariable domain. They also stated that once the T at bp 16189 is replaced by a C, the number of As and Cs in the domain becomes flexible, probably because of replication error. Quite often, this can cause length heteroplasmy and produce a blurred sequence after the C-stretch on

the electropherogram. This was observed on both the infant and relative's samples (Plate 2).

In point of fact, a careful examination of the sequence diagrams for the Cochrane case suggests that there are two bases instead of one on most of the sequencing results at position 16183 while only one base at the same position for the relative (Plate 2). Among the 12 sequencing results, only one indicates A at this position (Plate 2, E) and more than half show a predominant C over A (Plate 2, B,C). Theoretically, there are two interpretations: (1) that a contaminant individual contributed his or her DNA to the samples, and this could have been either the A or the C; or (2) that this represents a heteroplasmy (or mixed mitochondrial population due to cellular mutations) in the Cochrane individual. This means that each cell contained two types of sequences at this position. After carefully analysing the results, we can conclude that the Cochrane samples are heteroplasmic for position 16183. If contamination had occurred, we should expect that more than one sequence position would have mixed base pairs, but the same mixed sequence at just this one site was repeatedly obtained from DNA extracts. Also, we should not expect to see the same contamination in all of the DNA extracts from two different sets of tissue samples because they were treated using different decontamination techniques. Furthermore, if contaminant DNA were mixed in with the Cochrane sample solutions, then the contaminant individual would have to be an unknown because neither of these patterns matches with any of the field or laboratory workers. Finally, the contaminant individual would have to be a maternally, closely related person since both individuals still shared a unique pattern of three base pair differences with each other. A number of studies in the literature have clearly indicated from examinations of large family samples that heteroplasmy is much more common than was once thought (Holland & Parsons, 1999) and in particular, heteroplasmy in the hypervariable block of the HVI has been identified in a number of maternal lineages (Cavelier *et al.*, 2000). Studies have also reported a high frequency of A to C substitution at 16183 when T to C replacement occurs at 16189 (Bendall & Sykes, 1995; Malik *et al.*, 2002). Therefore, it should not be surprising

¹New data indicate that this may not be the case because a high mutation rate may produce frequent intergenerational differences (Holland & Parsons, 1999).

to observe a heteroplasmy A/C at 16183 from the Cochrane samples due to possible instability of the block caused by length heteroplasmy of the homopolymeric tract with 16189C (Bendall & Sykes, 1995).

It is interesting that a previous case of site heteroplasmy has been detected in a historical case. A heteroplasmy (16169 C/T) was found in the mtDNA of the purported skeletal remains of Russian Tsar Nicholas II but not in samples of his maternal living relatives. Initially, this heteroplasmy could not be convincingly distinguished from contamination (Gill *et al.*, 1994). As a result of this, the Russian government gave permission for the exhumation of the tomb of Grand Duke Georgij, the Tsar's brother, in order to take bone samples for DNA analysis. The two brothers matched for the same mixed base pairs of mtDNA sequences, but the relative amounts of each base are different (Ivanov *et al.*, 1996). One could ask why these heteroplasms would not be passed on to subsequent generations? Studies on animal models and large human families all indicate that heteroplasms do not persist for long but soon become fixed to one base over a few generations (Lightowlers *et al.*, 1997). We would assume then that in the mitochondria of the Cochrane infant, both A and C are present at site 16183 but that C became fixed in the living relative of the next generation.

Sex determination was also recognised to be an important part of the identification process of the Cochrane case. As noted above, due to the recovery of smaller amounts of DNA template and the higher risk of contamination for the first set of samples (a dedicated lab was not available at the time), a male result was obtained from the two positive results out of more than ten attempts at PCR amplification when a Y-specific sequence was targeted. This result was originally accepted because the majority of the mtDNA did not indicate any contamination. However, this result was problematic because the mtDNA analysis could not effectively monitor contamination of DNA sex estimation, since Y-specific DNA and mtDNA were amplified separately. Contamination may occur in one PCR setup, but not in others.

For the second set of samples, with better contamination controls and larger amounts of

tissue, more DNA template was extracted. Instead of using a single method for sex determination, three different methods were used: (1) the traditional 112bp/106bp amelogenin gene amplification (Sullivan *et al.*, 1993); (2) a preferential Y amplification procedure (Yang *et al.*, 1998b); and (3) a search for highly repetitive Y sequences (Hummel & Herrmann, 1994). Although there are challenges with each method when applied to archaeological or historical samples, the results indicate that the remains come from a female individual.

Discussion and conclusions

Positive identification of an individual who died in 1897 and was buried in a pioneer cemetery was achieved through the efforts of a multidisciplinary team. The archaeologist was able to define each grave carefully and to date the graves based on historical artefacts, such as nails, and the placement of graves relative to one another and to the former church. Provided with that information, the historian was able to identify particular families who used the cemetery and to obtain dates of death and placement of burial for particular family members. Together, the archaeological and historical information were crucial to the successful identification of the individual.

Based on that information, the human osteologists were able to apply particular methods of analysis (age determination from dental development and diaphyseal length, ageing by enamel histology, sex determination from DNA and testing of maternal relatedness through mtDNA analysis) in order to provide a positive identification. Morphological assessment, while not without error, is reasonably accurate for young infants. The dental age estimate of 3 months \pm 1 month is low by approximately one month. Similarly, age estimation from diaphyseal length, two to four months, also underages the individual by approximately one month. Age estimation from enamel microstructure provides a range from 4.8 to 5.1 months. The discrepancy in these age estimates may reflect nutritional stress suffered by the infant after birth. According to the historical documents, the infant's mother died

during childbirth and the child failed to thrive in the absence of maternal nurturing.

The results of the nuclear DNA analysis indicate that the individual was female. Analysis of the HVI region of mtDNA indicates four base pair differences when compared with the published reference sequence. These four differences are shared by the proposed living relative. While this does not constitute a positive identification in the legal sense, the congruence of archaeological, historical, morphological and molecular evidence points to one particular 5-month-old girl recorded in historical documents.

The living relatives were notified that the infant recovered from the former St. Mary's Church cemetery in Cochrane is a member of their family. Specifically, MEM is the aunt (mother's sister) of BF, now in her 80s. The remains of MEM were interred in the family plot in Calgary, where there is already reference to her on the family grave marker. This experience has been a very positive one for all involved. We established a good working relationship with the people of the community during the excavation. The town was extremely cooperative, as was the family, who invited three of us to attend the funeral.

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