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Ancient bacterial DNA (aDNA) in dental calculus from archaeological human remains

Hans R. Preus [a,*], Ole J. Marvik [b], Knut A. Selvig [c], Pia Bennike [d]

[a] Department of Periodontology, Institute of Clinical Odontology, Dental Faculty, University of Oslo, P.O. Box 1109 Blindern, N-0317 Oslo, Norway
[b] Affitech AS, Oslo Research Park, Oslo, Norway
[c] Dept of Anatomy, Faculty of Dentistry, University of Bergen, Norway
[d] Saxo Institute, University of Copenhagen, Denmark

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ABSTRACT

The purpose of this study was to identify reactive bacterial aDNA in archaeological human dental calculus. Dental calculus was collected from a middle/late Neolithic human skull from Hulbjerg passage grave, Langeland, Denmark and prepared for transmission electron microscopy (TEM) or gold-labeled antibody TEM. TEM showed calcified, as well as non-calcified bacteria. Immunogold labeling occurred over the cytoplasmic portions of the sectioned bacteria. The result demonstrated that it is possible to identify aDNA sequences from bacteria in archaeological material of considerable age by this technique.

1. Introduction

There is a growing interest for ancient biomolecules in several fields of science. Researchers are striving to develop more precise techniques to trace human migration and behavior in the past (Campana, 2008; Ricaut et al., 2004). Analyzing and comparing ancient DNA (aDNA) may also facilitate distinction between sites within an archeologically interesting area (Bollingono and Vigne, 2008; Fortea et al., 2008). Forensic scientists search for evidence of historic crimes through studies of (aDNA) (Schlablitsky et al., 2006; O'Rourke et al., 2000), and physicians use aDNA to study hereditary and infectious diseases in the past (Witas and Zawicki, 2006; Herrmann, 1998). It has also been suggested that studies of DNA from prehistoric bacteria or viruses may lead to the discovery of new approaches to fight modern versions of these infectious agents. A high-profiled example is the excavation and study of victims of the Spanish flu on the Island of Spitsbergen, Norway, in 1998 (Duncan, 2003; Annan et al., 2000). In this particular endeavor they searched for the Spanish flu virus DNA/RNA in order to study and produce antibodies against a future viral pandemic of similar origin. However, recent reports on current diseases, like tuberculosis and syphilis, have focused on historical information about the original phenotype and genetic development of the responsible bacteria (Hunniusvon et al., 2007; Hummel et al., 1995), also emphasizing the limitations of these techniques. The interest in the historic and original infectious agents is boosted by the growing concern that modern versions have evolved to escape the treatment of available antibiotic drugs (Høiby et al., 2010), and the desire to develop new antimicrobials based on the original DNA of the microorganisms.

A major problem in this field of research is the lack of specimens suitable for systematic analysis. Teeth and calcified material are identified in most, if not all, archaeological digs where human remains are found. Dental calculus is therefore a common find, and may serve as a systematic source of information in a field where only occasional findings of preserved aDNA have been the order of the day. The DNA from bacteria, fungi or viruses, and more seldom human cells, are obviously more preserved against contamination and deterioration embedded in amorphous calcifications than on the inner or outer surfaces of other human tissues. Studies of archeological calculus material have been reported (Gobetz and Bozarth, 2001; Herschkovitz et al., 1997; Middleton and Rovner, 1994), but to our knowledge, reporting it as a source of aDNA, has not been done.

The potential problem of historic and modern contamination of aDNA samples has been strongly emphasized by many researchers

* Corresponding author. Tel.: +47 22 85 21 63; fax: +47 22 85 23 96.
E-mail address: hpreus@odont.uio.no (H.R. Preus).

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(Bouwman et al., 2006; Gilbert et al., 2005; Yang and Watt, 2005; Pruvost and Geigl, 2004; Yang et al., 2003; Preus, 1998; Burger et al., 1997; Dutour, 1997; Schmidt et al., 1995). Thus the need for highly preserved aDNA sources, with less contamination potential, is of interest to the archaeological community.

In this communication we propose a way to overcome several of these obstacles, and suggest dental calculus as a systematic source of aDNA from archaeological material. Dental calculus contains bacteria and intercellular substance forming a matrix for deposition of amorphous and crystalline calcium phosphates and silicate (Lang et al., 2008). In this highly calcified substance the encapsulated bacteria are to a great extent protected against *post mortem* degradation, unless embedded in an acidic environment. Since nucleic acids are normally stabilized by divalent cat-ions, DNA is well preserved in calculus with its abundance of Ca^{2+} containing amorphous crystals. Moreover, as skulls and teeth are often the best preserved specimens in archaeological excavation sites, archeological dental calculus might be an ideal material for systematic studies of aDNA. Thus, the aim of the present investigation was to demonstrate the presence of reactive sequences of DNA from oral bacteria embedded in dental calculus from human remains of considerable age.

2. Materials and methods

2.1. Sample

Calculus was collected from the roots of the lower central incisors of a skull from the Stone Age Hulbjerg passage grave built in the Middle Neolithic on the island of Langeland, Denmark. The skull was found together with skeletal remains of 52 individuals including men, women and children. The archaeological artifacts and ^{14}C-analyses showed that the passage grave had been used periodically between 3200 and 1800 BC (Bennike and Bohr, 1990), indicating that the age of the calculus sample is between 4000 and 5000 years.

In passage graves, the bones of individual skeletons are usually scattered in anatomical disorder and are found in variable state of preservation. Some of the bones, like the skull from which the actual calculus samples were taken, were intact and well preserved, and the femora have been used in a study of bone mineral content in various periods (Skaarup, 1985). After the passage grave was closed, it has apparently remained undisturbed until its excavation in 1960. The skeletal material from the site was stored at the Laboratory of Biological Anthropology in Copenhagen. More recently an anthropological study has revealed evidence of both dental (a drilled hole) and surgical intervention (trephination) (Bennike, 1985). Dental calculus from the drilled tooth has previously been examined by scanning electron microscopy, revealing bacteria-like structures (Bennike and Fredebo, 1986). These specimens were different from those presented in the present article.

2.2. Methods

The dry calculus specimens from the root of the lower, central inscisor (2.1 Sample) were dehydrated in an aqua solution of graded alcohol, treated with osmium tetroxide fixative to enhance contrast, and embedded in epoxy resin following routine procedures for transmission electron microscopy (TEM). Sixteen specimens of either untreated sections, or sections treated with ultraviolet light and gold-labeled monoclonal antibodies specific for thymine dimers (Marvik et al., 1997), were processed for TEM. Thin sections were collected on electron microscopic grids and exposed to ultraviolet light irradiation (UV). Non-irradiated controls were also included. Moderate doses of UV at 310 nm will, in the presence of acetophenone, preferentially induce the formation of the 5′ 6′ cyclobutane type of thymine dimers, for which the selected H3 antibodies are specific (Roza et al., 1988). The H3 antibodies were produced by a mouse hybridoma cell line (Roza et al., 1988) that has previously been characterized for the use in photo-immunodetection (Marvik et al., 1997). They show a sensitivity in membrane assays of about 0.5 pg with a specificity of >10E6 higher affinity for irradiated versus non-irradiated samples. Thus for practical purposes they are unreactive toward DNA without 5′-6′ cyclobutane type of thymine dimers. Importantly, the reactivity of the H3 antibodies toward the 5′-6′ cyclobutane dimer decreases dramatically (>100 fold) when a thymine dimer is positioned closer than about 15 residues from the end of the chain (Marvik unpublished results; Roza et al., 1988). This makes the antibody a useful probe for irradiated DNA fragments larger than at least 20–30 residues. In short, series of 50 μl droplets of reactants and washes were prepared on sheets of parafilm. All grids were treated with each reactant by placing them inverted on the relevant series of droplets for the times indicated below. The grids were pretreated for 15 min with 2 mM acetophenone diluted 1:5000 in 1-x PBS. Subsequently, half of both the test and the control grids were irradiated with 80 kJ/m^2 in a Vilber Lourmat UV-crosslinker using tubes with peak emission at 310 nm. All grids were blocked with 10% inactivated Fetal Calf Serum (FCS) in 1-x PBS for 20 min before exposure to the anti-thymine dimer antibodies (either undiluted or diluted 1:10 in FCS/PBS) for another 20 min. Following washing on droplets of PBS for 5 times 5 min, the grids were treated with 10 nm gold-labeled secondary anti-mouse antibodies in 5% FCS/PBS for 20 min. Washing was repeated 4 times with PBS and 6 times with water and contrasted with 1% uranyl acetate with lead citrate before microscopy (Reynolds, 1963). Irradiated and non-irradiated sections of fresh mammalian connective tissue (dog gingiva), served as positive control. The experimental design is shown in Fig. 1.

3. Results

3.1. Transmission electron microscopy—untreated sections

Transmission electron microscopy of untreated calculus revealed a highly calcified substance with scattered voids corresponding to non-calcified bacteria (Fig. 2). Circular and elongated profiles within the calcified substance could be identified as calcified bacterial cells. Electron diffraction analysis indicated that the mineral crystals were predominantly hydroxyapatite with occasional, presumably *post mortem*, deposition of whitlockite in the voids.

3.2. Transmission electron microscopy—treated sections

The gold-labeled thymine dimer treated sections showed metal particles bound over the cytoplasmic portions of several non-calcified bacteria (Fig. 3). Eight clusters of 6–8 bacteria each were investigated by TEM, showing varying binding of particles from section to section. Gold particles were not observed in the intercellular matrix. The bacterial cell wall was distinctly outlined; however, the cytoplasm appeared shrunken and few or no ultra structural details were visible. Due to the high electron density of the mineral crystals, the presence or absence of labeling on calcified cells could not be determined.

3.3. Transmission electron microscopy—controls

The specificity of the reaction was verified by the presence of gold-labeled thymine dimer antibodies only over the nuclear