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(57) **Abrégé/Abstract:**

The present invention provides the use of animal calculus as a source of nucleic acid which is useful in genetic studies. Such genetic studies may centre on the host organism from which the calculus is derived or on microbial organisms whose nucleic acid is embedded in the calculus of the animal host.



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(21) International Application Number: PCT/GB98/01083 (22) International Filing Date: 14 April 1998 (14.04.98) (30) Priority Data: 9707775.4 17 April 1997 (17.04.97) GB (71) Applicant (for GB only): DZIEGLEWSKA, Hanna, Eva [GB/GB]; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB). (71)(72) Applicant and Inventor: PREUS, Hans, Ragnar [NO/NO]; University of Oslo, Faculty of Dentistry, Clinic for Periodonti, Postbox 1109, Blindern, N-0317 Oslo (NO). (72) Inventor; and (75) Inventor/Applicant (for US only): LILLEHAUG, Dag [NO/NO]; Agricultural University of Norway, P.O. Box 1109, Blindern, N-0317 Oslo (NO). (74) Agents: DZIEGLEWSKA, Hanna, Eva et al.; Frank B. Dehn & Co., 179 Queen Victoria Street, London EC4V 4EL (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: USES OF CALCULUS (57) Abstract The present invention provides the use of animal calculus as a source of nucleic acid which is useful in genetic studies. Such genetic studies may centre on the host organism from which the calculus is derived or on microbial organisms whose nucleic acid is embedded in the calculus of the animal host.		

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USES OF CALCULUS

The present invention relates to the use of calculus as a source of genetic information and in particular to the extraction and characterisation of microbial DNA from the dental calculus of long dead mammals.

Calculus is a solid mixture of mineral salts, mainly calcium based, which builds up on the surface of teeth and certain other organs, but it may also contain phosphorus, magnesium, fluorine, cobalt, silicon and sodium (Rølla, G. et al., (1989) in Recent Advances in the Study of Dental Calculus Ed. J. M. ten Cate, IEL Press pp 123-126; Glock, G. E. and Murray, M. M. (1938) J. Dental Res. 17:257-264). Dental calculus also comprises an organic phase of bacteria and bacterial remnants, cell products and various compounds found in saliva and gingival fluid (Eggen, K. H., and Rølla, G. (1985) Scand. J. Dent. Res. 93:426-431). Dental calculus may occur supra- and sub-gingivally and there may be differences in the abundance, distribution and structure between the types.

Calculus is found in the ducts, passages, hollow organs, cysts and the surfaces of teeth in humans and other mammals. Calculus persists for many years after the death of the organism and well preserved calculus has been detected, for example, on the teeth of bodies in excess of 5,000 years old. In contrast, all the soft tissues of an organism decompose unless the body is mummified, frozen or otherwise preserved.

Other than its mere existence, most notably on teeth, little is known about the inorganic matrix which constitutes calculus. It is formed by a non-enzymic accumulation of dental plaque, calcium and other mineral salts on a surface which eventually becomes calcified. Nucleators ie. organic or inorganic structures which may

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serve as templates for deposition of calcium phosphates, are believed to be an important factor in calculus formation and plaque bacteria may play a role. It is widely regarded as an amorphous matrix and most scientific and clinical interest in calculus is directed towards its prevention and removal, since an accumulation of calculus on the teeth is cosmetically undesirable and can result in inflammation of the gums leading to dental problems (Mandel, I. D. and Gaffer, A. (1986) J. Clin. Periodontol. 13:249-257).

It is generally accepted that dental calculus forms to a greater or lesser extent on all mammalian teeth. A major clinical feature of calculus is that it binds tenaciously to the teeth and is difficult to remove. Its formation is spontaneous and effected by diet, chewing behaviour and the micro-environment of the mouth in particular pH. Additionally, there may be some genetic predisposition towards the greater or lesser formation of calculus and certain ethnic groups e.g. Asians, are particularly affected.

pH is a determining factor in the type of calcium salts which predominate in calculus. Animals with a salivary pH of around 8 e.g. dogs and pigs exhibit dental calculus comprising mostly calcium carbonate. Animals with a lower salivary pH of 7, for example, primates including man, exhibit dental calculus based mainly on calcium phosphate deposits (Driessens, F. C. M. & Verbeeck, R. M. H. (1989) In Recent Advances in the Study of Dental Calculus Ed. J. M. ten Cate, IRL Press pp. 7-17).

Similarly, humans with high urinary pH develop urinary calculi comprising mainly calcium carbonate; those with neutral urine develop calculi comprising calcium phosphate and individuals exhibiting low pH urine, who develop calculi, will produce stones rich in uric acid salts.

Diet can affect the formation of calculus in

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different ways, for example, hard, abrasive foods are believed to assist in preventing calculus formation on the teeth by mechanical agitation.

Dietary silicon can, under certain circumstances promote the formation of urinary calculi in cattle and sheep and there is some suggestion that a high silicon diet, particularly from rice, correlates with increased formation of dental calculus (Gaare, D. (1989) J. Dent. Res. 68:(Spec Issue)1710-1711; Rølla et al., 1989).

It has recently been shown that calculus on the teeth of both living and long dead people, contains the calcified remains of microorganisms embedded in its matrix. This has not been well researched, but the number and nature of the microorganisms trapped within the calculus appears to be variable. Most bacteria occurring in dental calculus are expected to be plaque bacteria (for example *Streptococci* and *Veillonella*) or microorganisms abundant in the buccal flora of the subject. Often, only a single microorganism has been detected, embedded in the calculus matrix.

Surprisingly, not least since microorganisms are present in calculus in such low and variable numbers, it has now been shown that genetic material can be extracted from the calculus of bodies which have been dead for as long as 5,000 years. Even more surprisingly, the isolated nucleic acid, for example from calcified microorganisms embedded in dental calculus, may be isolated in a form amenable to manipulation by recombinant DNA technology and thus may be amplified, sequenced, cloned, expressed, mutated and otherwise manipulated by any molecular biology technique known in the art. Such amenability to manipulation may thus facilitate the study of gene sequences of microorganisms which lived many thousands of years ago.

Although the isolation of genetic material from cells embedded in calculus on archaeological material is a primary feature of the invention, calculus from living

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animals may also be used as a source of nucleic acid for genetic study.

Whereas dental calculus is believed to harbour only or primarily preserved microbial cells, calculus from other body parts, for example urinary calculi, may also harbour remnants of preserved host cells. In the case where the host has been subjected to infection or where the calculus forms in a body part normally colonised by microorganisms, the calculus may provide a record of host DNA and also DNA from any pathogen, saprophyte or commensal organisms to which the host had been exposed during its lifetime.

Thus, according to one aspect, the present invention provides the use of animal calculus as a source of nucleic acid.

In particular, the invention provides a useful source of nucleic acid for genetic study.

The calculus may be obtained from any animal body on or in which it is laid down. Conveniently however, the animal will be any mammalian animal.

The calculus may be isolated from any part of the animal, preferably mammalian body.

The nucleic acid may be of host or foreign, eg. microbial origin, and may be DNA or RNA. Preferably, however, it is DNA. More preferably if the DNA is of eukaryotic origin, it is nuclear DNA.

In a preferred aspect of the invention, the genetic material is from microorganisms which may be isolated from calculus, formed during the life of the host, on or in any part of the body. A particularly advantageous feature of the present invention is that the microbial material isolated from the calculus, may comprise ancient microorganisms. In other words the host body, from which the calculus is obtained may have been long dead. Thus, the calculus may be obtained not only from intact bodies which may be dead or alive but also from skeletal remains.

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The invention thus encompasses the use of a living animal body as a source of the calculus, or a dead body, which may be recently or long dead. The dead body may be intact in its entirety, or body parts may be used, with
5 varying degrees of decomposition. Skeletal material devoid of soft tissue may be used. "Long dead" as used herein encompasses anything from tens to thousands of years from death. In other words, the body or body part, from which the calculus is obtained, may have died from 1 day to up
10 to 20,000 years or more ago, and preferably, up to 10,000 years ago. The invention thus lends itself well to the study of archaeological or archive material.

In a particularly preferred aspect, the calculus from which the nucleic acid is isolated is dental calculus.

15 In another aspect, the invention provides the use of dental calculus from an animal as a source of nucleic acid, wherein said nucleic acid is obtained or isolated directly from calcified microbial cells or cellular material embedded in said calculus, or wherein said nucleic acid is
20 derived by copying or amplifying the said directly isolated nucleic acid.

In another aspect, the invention provides a method of obtaining microbial nucleic acid characterised in that said nucleic acid is obtained or isolated directly from
25 calcified cells or cellular material from microbial organisms embedded in dental calculus from an animal or is derived by copying or amplifying said directly isolated nucleic acid.

In another aspect, the invention provides a method
30 of studying microbial genetics, characterised in that microbial nucleic acid for said study is obtained or

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isolated directly from calcified microbial cells or cellular material embedded in dental calculus from an animal or is derived by copying or amplifying said directly isolated nucleic acid.

5 In another aspect, the invention provides a method of comparing nucleic acids from different individuals, characterised in that the nucleic acids are obtained or isolated directly from calcified microbial cells or cellular material embedded in dental calculus from an animal or are
10 derived by copying or amplifying said directly isolated nucleic acids.

 In another aspect, the invention provides a process for the preparation of an isolated nucleic acid of microbial origin, wherein nucleic acid is obtained or
15 isolated directly from calcified microbial cells or cellular material embedded in dental calculus from an animal or derived by copying or amplifying said directly isolated nucleic acid.

 Alternatively viewed, a preferred aspect of the
20 invention can thus be seen to provide a method of obtaining microbial nucleic acid, characterised in that the said nucleic acid is derived from microorganisms contained in the calculus of an animal host.

 The term "microorganism" is used within the
25 context of this application to cover all microbial organisms or cellular material and in particular bacteria, fungi and viruses as well as plant cells such as algae including blue-green algae, and protozoa. Spores from sporulating bacteria or fungi are also within the scope of the term microorganism
30 as are plasmids, bacteriophage and mycoplasmas.

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In terms of microorganisms associated with the mammalian body, they may be of any species or genus and of course may be commensals, pathogens or saprophytes.

Although this represents a preferred aspect, the
5 invention is not limited to isolating the nucleic acid of microorganisms from calculus, and the nucleic acid may be obtained from any cells or cellular material eg. organelles which are contained in the calculus,

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including all eukaryotic cells which may be plant or animal cells or protoplasts, and include the cells of the host.

Genetic information gleaned by the study of nucleic acids obtained from calculus is very useful to the pharmaceutical industry, by providing sequence data from microorganisms which existed prior to the advent of antibiotics, biocides or other means for the control of microbial activity and hence prior to the emergence of antibiotic resistance. Such information may add to the understanding of the evolution of resistance mechanisms and augment the development of new strategies and formulations for overcoming and/or avoiding the development and spread of drug resistance.

It is believed that ancient microorganisms themselves produced substances, for example metabolites or enzymes, with antimicrobial properties which provided them with a competitive advantage against other microorganisms. Identifying such products from expressing cloned genes from the ancient microorganisms, may provide novel anti-microbial products, useful in themselves. It will be understood by the skilled person that such novel gene products may find many applications in a diverse range of scientific areas. Of particular interest however is the potential such compounds have for counteracting multiple drug resistance in present day pathogenic organisms which will never have been exposed to such compounds.

Replacement therapy is viewed by many as being a new approach to combatting infection and the use of genetic information from calculus derived microorganisms could provide significant advances in this regard.

As mentioned above, calculus may form and harbour microorganisms on body parts exposed to microbial infection, or in areas normally colonised by microorganisms. Thus, genetic information about microorganisms with specific habitats or infection sites

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for example, coliforms or enteric pathogens in the gastrointestinal tract, *Streptococci* or *Mycobacteria* in the lungs or *Staphylococci* in the urinary tract may be generated and the information used by the pharmaceuticals industry in the avoidance of antibiotic resistance, replacement therapy and for the expression of novel products as described above.

Furthermore, it is thought that insofar as dental calculus is concerned, microorganisms responsible for many disease processes, not only those limited to the buccal cavity or gastrointestinal tract, may be represented in the calculus matrix.

In addition to the commercial, pharmaceutical benefits provided by use of the present invention, the information gained from calculus-derived microbial genetic information may be useful in the study of population movement and interaction in groups of people and animals thousands of years ago.

Also, it may provide answers to many questions which still surround ancient epidemiological riddles. For example, it is still not certain which microorganism(s) was the cause of the "black plague" which killed large numbers of people in continental Europe in the 12th century. Since the soft tissues of all the people killed by the plague were actively destroyed, there is no identifiable repository of the organisms which killed the victims available for studies. It is proposed herein however, that study of the organisms and genetic material therein, embedded within the dental or other body calculus of the victims of the plague or other diseases, will yield an identity and characteristics of the microorganism(s) responsible.

In addition to the clear scientific and anthropological interest this would generate, such studies may also yield valuable epidemiological data useful for today's understanding of the spread of disease within populations and therefore provide means for its

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circumvention.

In a further preferred aspect therefore, the calculus from which microbial DNA is extracted is of human origin.

A particular advantage of using dental calculus as a source of microbial DNA is that it does not contain significant amounts of host DNA and hence the study of ancient microbial DNA is not complicated by host DNA contamination.

The resilience of calculus to degradation means that even on body remains which have been burnt and subject to exposure to the elements for millennia, there is effectively no age limit to the samples which may usefully provide genetic information.

Viewed from a further aspect, the present invention provides a method of study of microbial genetics characterised in that microbial nucleic acids for said study are derived from animal calculus.

The term "genetic study" is used herein in its broadest sense to encompass all forms of study or analysis related to the genome, including not only epidemiological studies and comparisons between different organisms, or organisms obtained from different sources of calculus eg. animals from different locations or of different species, but also any study or manipulation of the isolated genetic material itself eg. sequencing, cloning, expression, modification, amplification etc. Many techniques for genetic study are described for example by Sambrook et al., in "Molecular Cloning", a Laboratory manual, 2nd Edition, Cold Spring Harbour Laboratory Press, 1989.

The term "derived" is used herein to include not only obtaining or isolating the nucleic acid directly from the calculus, but also nucleic acid derived by copying or amplifying the originally isolated source nucleic acid.

Also provided according to the invention is a

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method of comparing nucleic acids from different individuals, characterised in that the nucleic acids are derived from animal calculus. Preferably, the nucleic acids are derived from microorganisms within the calculus.

The "individuals" may be any animal, but advantageously will be human. The nucleic acids derived from the calculus may be compared using any of the techniques for genetic study which are well known in the art and widely described in the literature eg. by *in vitro* amplification eg. by PCR and subsequent analysis of the amplification products eg. by size fractionation by gel electrophoresis, restriction fragment length polymorphism analysis (RFLP), hybridisation-based methods, sequencing, etc.

Presently, where mummified or frozen remains of soft tissues exist, it is possible to identify and study genetic information from the host and also any organisms remaining in the soft tissues. Where no soft tissue remains exist however this is clearly not possible. If however calcified material can be identified within the body cavity ie. calculus, it would be possible using the present invention to identify and characterise the genetic information from both the host and any microorganisms present.

Hence, in a further aspect, the present invention provides the use of calculus originating from an animal host body as a source of genetic information derived from the host. The calculus may conveniently be from the ducts, passages, hollow organs and cysts which form within the animal body. Where the host animal has been subject to infection or normal microbial colonisation, calculus derived from tissues such as e.g. the ducts, passages, hollow organs and cysts may further provide a source of genetic information of those organisms.

The methods employed in the extraction, characterisation and molecular manipulation of the

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extracted genetic information may all be performed by techniques used in the art and a process for the preparation of isolated nucleic acids of host or microbial origin is provided herein, characterised in that the nucleic acids are derived from animal calculus.

One possibility for extracting nucleic acids from calculus involves isolating the calculus from the body and washing it with antimicrobial agents to remove surface contamination. The calculus is then pulverised and the nucleic acid trapped therein is eluted by water. A similar technique for extracting DNA from bone and teeth has been previously described by Petrischev, V. N. *et al.*, (1993) *Genetika* 29;690-693.

Another approach is to subject the washed calculus to a process of demineralisation by suspending the isolated calculus in an aqueous solution comprising EDTA, or a similar chelator of divalent cations, which binds calcium ions, the major substituent of calculus, and magnesium ions. Chelation of magnesium ions inhibits DNase enzyme activity which would degrade the DNA. A similar approach has been described by Hall, R. C. *et al.*, (1996) *Eur. J. Oral Sci.* 104:285-291, in the extraction of native DNA from teeth.

Alternatively, it has been shown by Fisher, D. L. *et al.*, (1993) *J. Forensic Sci.* 38:60-68, that it is possible to apply DNA extracted from undecalcified material directly to a PCR reaction mixture. Furthermore, the yield of DNA extracted from undecalcified material was double that from decalcified material. An analagous process may be applied to calculus.

DNA within the calculus may be amplified *in situ* using PCR. The resultant fragments may be hybridised *in situ* and visualised or may be extracted and purified. This technique involves sectioning the calculus in a similar manner to that used for electron microscopy and then creating a PCR reaction chamber on a glass slide by

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adding the PCR mixture to the section under analysis and enclosing the mixture using a rubber seal. The PCR reaction may then be carried out as appropriate.

Silica based purification methods as, for example, described by Hoss, M. & Paabo, S. (1993) *Nucleic Acids Res.* 21:3913-3914, may also be used successfully in the extraction of nucleic acid from calculus.

Yet a further possibility may be the breaking down of silicate junctions which form the cornerstones of calculus by using weak acids and extracting the nucleic acids therefrom.

Other methods which may be used or adapted for use in extracting nucleic acids from calculus include the procedures discussed by Cattaneo, C. et al., (1995) *Forensic Sci. Int.* 74:167-174; Laitinen, J. et al., (1994) *Biotichniques* 17:316, 318, 320-322; Simpson, T. A. & Smith, R. J. (1995) *Laryngoscope* 105:28-34. Any method described in the literature, for example, for extracting nucleic acids from bones or skeletal material could be used, for example, techniques which break down silicate junctions of calcified material and of particular interest are techniques specifically adapted for use with ancient material, as described for example, by Handt, O. et al., (1994) *Experimentia* 50:534-529, or Woodward, S. R. et al., (1994) *PCR Methods Appl.* 3:244-247.

Thus, although the nucleic acids may be extracted by any known methods, methods in which an appreciable amount of nucleic acid is lost during the extraction and purification process for example, those involving solvent extraction e.g. phenol/chloroform extraction, precipitation for example using precipitants such as salts, or the binding of nucleic acid to various types of matrices are less preferred, since it is desirable to perform the purification with as little loss as possible.

It is desirable that the methods employed to

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extract and purify the nucleic acid should result in minimal amounts of degradation and/or loss of DNA. This is important since the amount of DNA or other nucleic acid available for study may be small.

In combination with any extraction method employed, it may be advantageous to disrupt any microbial cell walls which remain intact within the calculus matrix. This may be done via a series of boiling and freezing (in liquid nitrogen) steps or by using enzymic degradation, for example lysozyme and/or mutanolysine and a proteinase such as Proteinase K. Other methods exist in the art which are suitable for removing or disrupting cell walls or achieving cell lysis as will be easily recognised by the skilled person.

Once in solution, residual enzymes may be inactivated by boiling and salts, for example EDTA, may be removed by dialysis or similar procedure if desired. The DNA may then be concentrated by dehydration. Optionally, extracted nucleic acids may be run through an agarose gel by electrophoresis prior to subjecting them to PCR or other manipulations, since this has the effect of removing most inhibitors of the PCR or other reactions. Clearly, this may not always be possible when working with very small samples.

PCR amplification and any other techniques known and used within molecular biology may then be used to amplify, sequence, clone, manipulate and express the DNA. PCR primers, for example, suitable for species identification and classification may be designed and the purified DNA applied directly to a PCR reaction mixture. The resultant products of the PCR reaction may then be sequenced to allow identification and initial characterization of the origin of the DNA in question. Any of the techniques known in the art and described in the literature for the manipulation or detection of nucleic acids may be used, including in addition to any of the *in vitro* amplification methods or sequencing

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methods, methods for detecting point or other mutations, base transitions etc.

In addition to extraction, amplification and manipulation procedures of chromosomal DNA, human genetic information derived from host cell remnants are amenable to techniques such as mitochondrial DNA sequence analysis.

Alternatively, nucleic acid may be studied *in situ* ie. directly on the calculus material, by sectioning the calculus using micro-manipulation techniques, or it may be studied by extracting the nucleic acid from individual cell remnants *per se* and then purifying it.

The invention will now be described in more detail by way of the following non-limiting examples with reference to the drawing in which Figure 1 shows an electron micrograph of a section of calculus taken from the teeth of a 5,000 year old body, believed to be that of an ancient Norwegian farmer. The round structures are calcified microorganisms. Magnification is 10,000X.

Example 1

Dental calculus was isolated mechanically from the teeth of a 5,000 year old non-mummified skeleton, thought to be that of a farmer. The calculus was washed in an antimicrobial solution to clean the surface, crypts and ducts and remove contaminants. It was washed again and then sectioned using standard techniques for electron microscopy studies. Electron micrographs show bacteria embedded within the calculus (see Fig. 1). The bacteria within the calculus are observed as round bodies and the morphology of the microorganisms is well preserved.

Example 2

Extraction of DNA from the calculus

The calculus is carefully scraped from the surface of the teeth and washed as described in Example 1. The

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calculus is suspended in 250 mM EDTA (pH 8.0) containing 20 mg/ml lysozyme and 1200 units/ml mutanolysine. The mixture is incubated for 30 minutes at 37°C and then SDS and proteinase-K are added to a final concentration of 0.1% and 50 µg/ml, respectively. The mixture is incubated for a further 30 minutes at 65°C. The mixture is subjected to 5 sequential cycles of boiling for 1 minute followed by freezing in liquid nitrogen for 1 minute. The resultant liquid is dialysed for 30 minutes on a filter against a 10 mM tris buffer (pH 8.0; on a petri-dish). The nucleic acid containing liquid is transferred to an Eppendorf tube and the nucleic acid dehydrated by vacuum (in a "speedvac"). The dehydrated samples are resuspended in an appropriate volume of dH₂O (5-20 µl).

DNA prepared by this method may then be applied directly to PCR reaction mixture for PCR amplification. Resultant strands of DNA may be separated by agarose-gel-electrophoresis and purified therefrom by standard methods and the purified DNA sequenced.

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CLAIMS:

1. The use of dental calculus from an animal as a source of nucleic acid, wherein said nucleic acid is obtained or isolated directly from calcified microbial cells
5 or cellular material embedded in said calculus, or wherein said nucleic acid is derived by copying or amplifying the said directly isolated nucleic acid.
2. The use as claimed in claim 1 wherein said nucleic acid is used for genetic study.
- 10 3. The use as claimed in claim 2 wherein said animal is mammalian.
4. The use as claimed in claim 3 wherein said mammal is a human being.
5. The use as claimed in any one of claims 1 to 4
15 wherein said nucleic acid is DNA.
6. The use as claimed in claim 5 wherein said DNA is nuclear DNA.
7. The use as claimed in any one of claims 1 to 6 wherein said calculus is formed during the lifetime of said
20 animal.
8. The use as claimed in any one of claims 1 to 7 wherein said animal has been dead for up to 20,000 years.
9. The use as claimed in claim 8 wherein said animal has been dead for between 500 and 10,000 years.
- 25 10. The use as claimed in either of claims 8 or 9 wherein said animal exists as skeletal remains.

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11. The use as claimed in any one of claims 1 to 10 wherein said microbial cells or cellular material are from an organism selected from any one of the following: bacteria, fungi, viruses, plant cells, algae, blue-green
5 algae, protozoa, spores, bacteriophage and mycoplasmas.

12. The use as claimed in claim 11 wherein said microbial organism is associated with a disease process.

13. The use as claimed in claim 12 wherein said organism is selected from any one of the following:
10 coliforms, enteric pathogens, *Streptococci*, *Mycobacteria* and *Staphylococci*.

14. The use as claimed in any one of claims 1 to 13 wherein said nucleic acid encodes anti-microbial substances or products associated with microbial resistance mechanisms
15 to anti-microbial products.

15. The use as claimed in any one of claims 2 to 14 wherein said genetic study is microbial epidemiology.

16. A method of obtaining microbial nucleic acid characterised in that said nucleic acid is obtained or
20 isolated directly from calcified cells or cellular material from microbial organisms embedded in dental calculus from an animal or is derived by copying or amplifying said directly isolated nucleic acid.

17. A method of studying microbial genetics,
25 characterised in that microbial nucleic acid for said study is obtained or isolated directly from calcified microbial cells or cellular material embedded in dental calculus from an animal or is derived by copying or amplifying said directly isolated nucleic acid.

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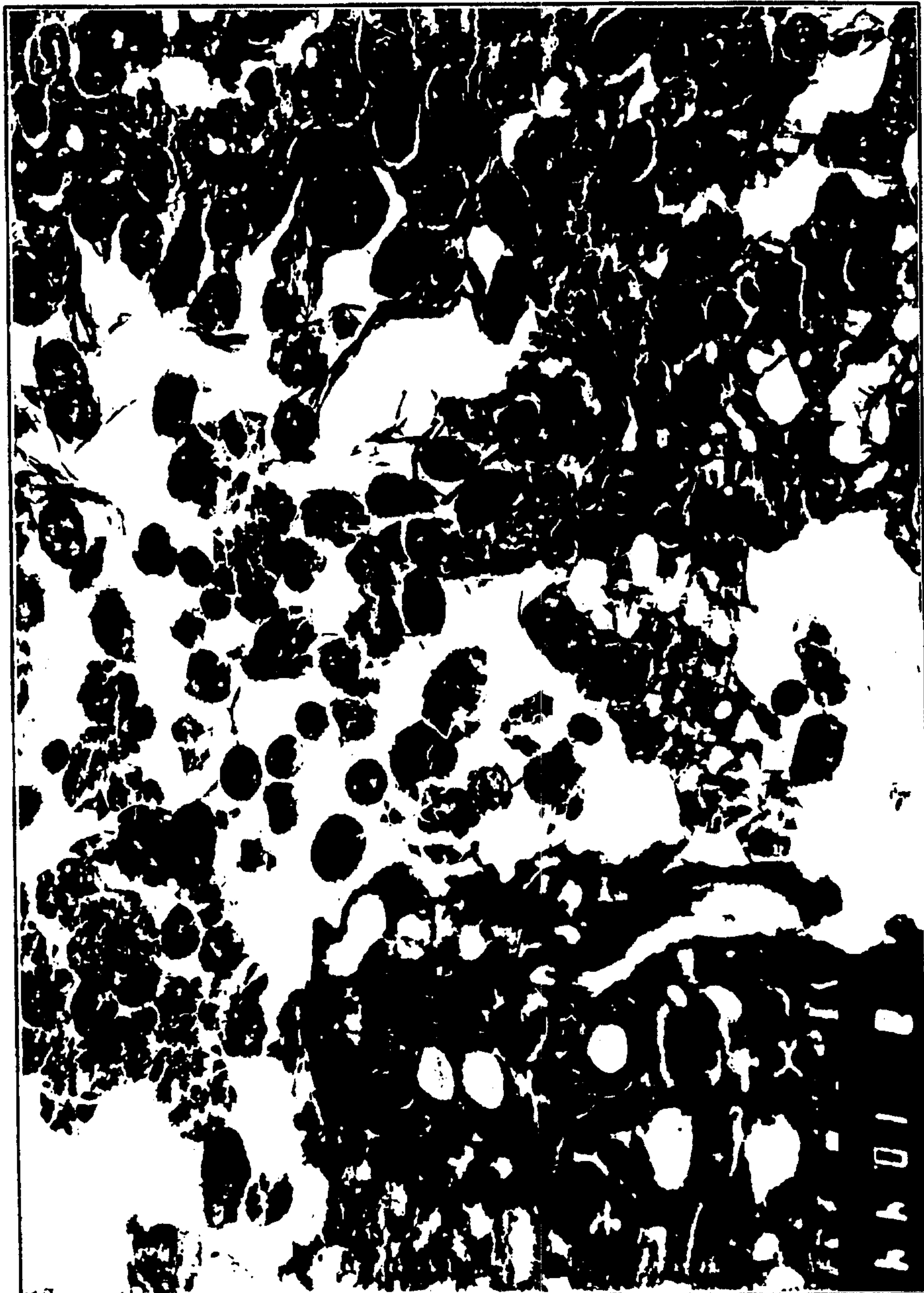
18. A method of comparing nucleic acids from different individuals, characterised in that the nucleic acids are obtained or isolated directly from calcified microbial cells or cellular material embedded in dental calculus from an
5 animal or are derived by copying or amplifying said directly isolated nucleic acids.

19. A process for the preparation of an isolated nucleic acid of microbial origin, wherein nucleic acid is obtained or isolated directly from calcified microbial cells
10 or cellular material embedded in dental calculus from an animal or derived by copying or amplifying said directly isolated nucleic acid.

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PATENT AGENTS

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