Chapter 11
Dental Pulp as a Tool for the Retrospective Diagnosis of Infectious Diseases

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Abstract Dental pulp is a highly vascularised tissue of mesenchymal origin, located inside the tooth and naturally protected from the external environment. As with any soft tissue, dental pulp might contain microorganisms that circulate in the bloodstream. Therefore, dental pulp has been used as a tool for the detection of septicaemic infectious agents in both contemporary and ancient human and animal specimens. This chapter reviews the different methods used for the detection of microorganisms in dental pulp, most notably DNA-based methods. We propose a protocol for the use of dental pulp as a tool for the molecular detection of blood-borne microorganisms. This protocol would be useful for retrospective diagnosis of infectious diseases in palaeomicrobiology.

11.1 Introduction: What is Dental Pulp?

Dental pulp is a soft tissue of mesenchymal origin that occupies the central cavity and the root canals of teeth. The outermost layer in healthy pulp is the odontoblast layer of cells, which is located immediately below the predentin. The middle layer is a cell-poor zone in the coronal pulp that is sometimes not visible in young individuals; the inner layer is a cell-rich zone forming the dental pulp itself (Fig. 11.1). Dental pulp is a well vascularised tissue with arterioles entering the tooth through the apical foramina and accessory canals and passing centrally through the pulp, giving off lateral branches and dividing into capillaries. Smaller vessels reach the odontoblasts, where they divide extensively to form a plexus below and within the odontoblastic layer. Venous return is ensured by a network of capillaries that merge to form venules coursing down the central portion of the pulp. The vessels in dental pulp are of the terminal vascularisation type and have a density similar to

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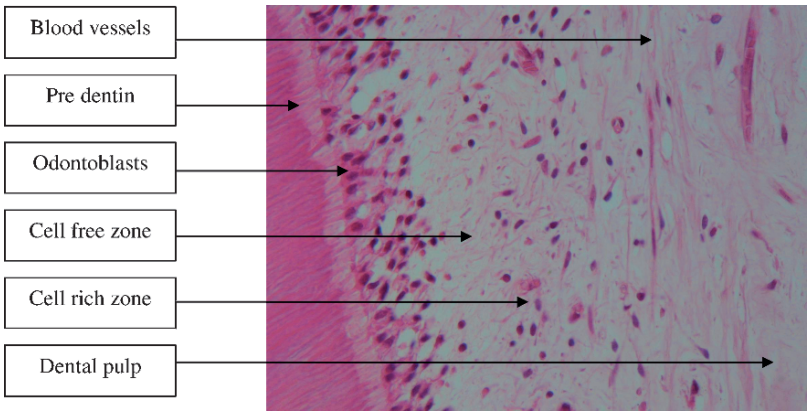

Fig. 11.1 Histology of mature human dental pulp. (Photograph: Dr. Marie Jos, Marseille Dental School, Marseille, France)

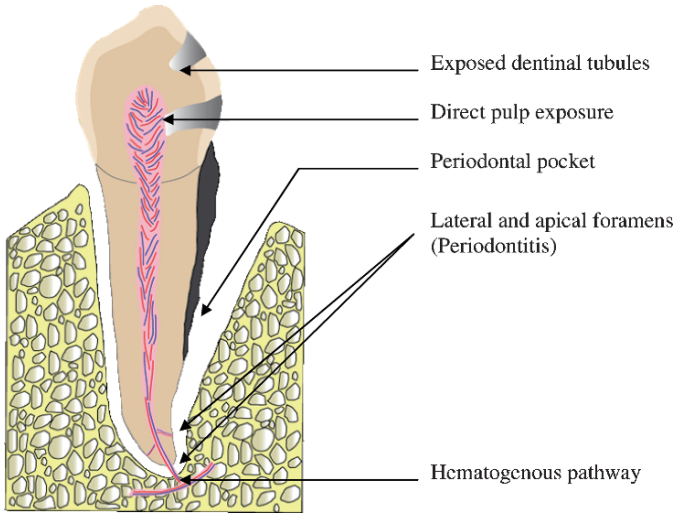

Fig. 11.2 Pathways of pulpal infection

that of vessels in the human brain (Kim 1985). Therefore, if an animal or a person has died due to a bacteraemia, it might be possible to find the pathogen responsible in the dental pulp. Changes that occur in dental pulp during life depend on the type of dentition growth. In rodents, there is continuous growth dentition, where hard dental tissues are continuously produced in order to replace those lost during chewing. The roots are open and there is usually a large volume of dental pulp. In other mammals, there is limited growth of dentition and, when teeth erupt, they are at

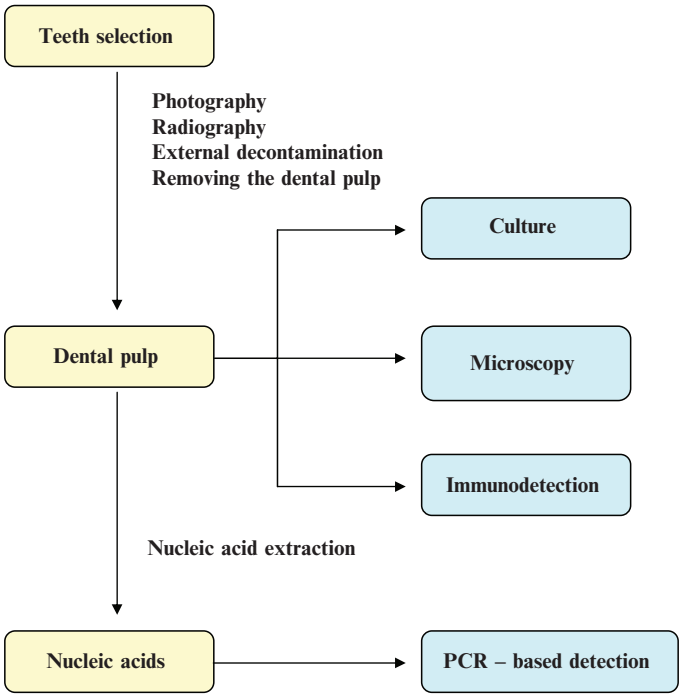

Fig. 11.3 Methods used for the detection of microorganisms in dental pulp

their final size, although the roots form completely only later. After the apical roots close, the pulp volume decreases gradually over time. Dentinogenesis is the inward formation of secondary dentin and is a continuous process. In people, continued growth of secondary dentin throughout life gradually reduces the size of the pulp chamber and the root canals. The dental pulp is protected from the environment by the surrounding dentin and enamel in the crown or cement in the root. Dental pulp can be infected by different routes, i.e. exposed dentinal, direct pulp exposure or via lateral or apical foramen and blood-borne microbes (Kettering 1994) (Fig. 11.2). This chapter focusses on the evidence for dental pulp infections resulting from blood-borne microorganisms and its application to the retrospective diagnosis of infectious diseases.

11.2 Methods Used for the Detection of Microorganisms in Dental Pulp

Methods used for the detection of microorganisms in dental pulp are summarised in Fig. 11.3.

11.2.1 Microscopy

The first study of bacterial colonisation of dental pulp in intact teeth was made by Tunnicliff and Hammond (1937). After disinfecting intact teeth and proving the sterility of their outside surfaces, dental pulp specimens were removed using sterile, fine-curved forceps and divided using sterile scissors. Smears were stained with gentian violet and Giemsa. The dental pulp was fixed in 1% formalin and sections stained with Gram–Weigert solution, hematoxylin-eosin and carbon thionin. Histology showed cocci and bacilli in different parts of the pulp without leukocyte infiltration. This was the first study of bacterial colonisation of dental pulp without dental irrigation (usually by preparing cervical cavities in teeth). In a second study (Tunnicliff and Hammond 1938), cavities were prepared in the teeth of dogs, which were intravenously injected with *Escherichia coli* and group A *Streptococcus*. After their removal, the teeth were fixed in 10% formalin or cold acetone, decalcified in 5% formic acid and embedded in paraffin. Six micrometric sections were cut and stained using the modified Brown and Brenn method (Brown and Brenn 1931). Bacteria were microscopically observed in the dental pulp of teeth removed at different times post-infection. Similar results were obtained by Tziafas (1989).

11.2.2 Immunodetection

The only immunodetection study of bacterial colonisation of dental pulp to date was performed by Gier and colleagues in 1968 in dogs (Gier and Mitchell 1968). Deep cavities were prepared in 88 teeth from four dogs, which were then inoculated intravenously with *E. coli*. A small piece of absorbent paper saturated with croton oil was sealed beneath a thick mixture of zinc oxide and eugenol in half of the cavities and the other half was left open to oral fluids. Bacteria were found in histological sections of unexposed pulp in 48 of 67 teeth in both prepared types of cavity and confirmed by culture. However, bacteria were also found in some blood vessels, but not in the pulpal tissue of one of the control teeth (unprepared) that was harvested 30 min after inoculation of bacteria. The authors remarked that the inflammatory reaction of the pulp was proportionate to the degree of injury and that bacteria were not found in 23 uninjured control teeth.

11.2.3 Culture

In a study in guinea pigs, Aboudharam et al. (2004a) prepared deep cervical cavities and injected bacteria intravenously. Of 15 unexposed dental pulps, 11 yielded group A *Streptococcus* and *E. coli* in culture. Similarly, culture of dental pulp yielded *Coxiella burnetii* in 2 of 12 samples after experimental bacteraemia in guinea pigs. No cavities were prepared and the cultures were positive at days 7 and

21 postinfection. These results suggested that dental pulp was equivalent to a small blood sample for the recovery of pathogenic agents. Viable *C. burnetii* were found in the dental pulp of guinea pigs after a week-long bacteraemia had ended (Aboudharam et al. 2004a). The authors suggested that dental pulp was a sanctuary for *C. burnetii* but the relevance of this finding to patients is unclear; guinea pigs have continuously developing dentition with open apexes, which is very different to the situation in human patients.

11.2.4 Nucleic-Acid-Based Detection

Nucleic-acid-based detection is the method most commonly used in this type of biomedical research. Nucleic acids extracted from dental pulp can be used for genomic amplification, most often by PCR, followed by sequencing and identification. This method has been used to make retrospective diagnoses of infectious diseases by detecting specific microbial sequences in dental pulp (Aboudharam et al. 2004a, 2004b, 2005; Drancourt et al. 1998, 2004, 2005; Glick et al. 1989; La et al. 2004, 2005; Papagrigorakis et al. 2006; Raoult et al. 2000; Wiechmann and Grupe 2005). In these studies, teeth were decontaminated, the dental pulp was removed by different methods, and PCR performed to search for specific fragments of microbial genomes. Such experiments have to be carefully controlled at each step because the method is highly sensitive and there is a high risk of contamination resulting in false positive results. We developed different protocols to minimise external contamination, including suicide PCR (Raoult et al. 2000) and carrying out experiments in laboratories where the bacteria of interest had never been introduced or worked on (Drancourt et al. 2004). We also modified a protocol initially proposed by Gilbert (Gilbert et al. 2003) whereby sterile auto-polymerisation resin was used to recover dental pulp without directly exposing it to the environment (La et al. 2005). All of these efforts (detailed in Sect. 11.3) helped to reduce the risks of contamination and to authenticate results. This approach is very useful in the study of ancient specimens where DNA has been shown to be better conserved in dental pulp than in bone (Ricaut et al. 2005), the most popular specimen used for the study of ancient DNA.

11.3 Detection of Infectious Agents in Dental Pulp During Bacteraemia

11.3.1 Experimental Models

Microbial colonisation of dental pulp was first studied in teeth with provoked inflammation. Early studies were performed in the 1940s–1960s and the target tissues in these studies were irrigated by preparing cervical cavities. The results,

determined by microscopic or histopathological examination, did not distinguish infections arising from bacteraemia or from infections with oral bacteria because the dental pulp may be exposed via dentinal tubules when deep cavities are created in teeth. Delivanis further demonstrated that organisms did not appear in fluid collected following bacteraemia from the dental pulp of canine teeth that had no blood circulation (Delivanis et al. 1981). This phenomenon was recently confirmed by electron microscopy of dental pulp collected 1 day after a suspension of streptococci was injected intravenously in dogs (Tziafas 1989). These data indirectly suggested that, during bacteraemia, microorganisms can reach the dental pulp in cases of previous dental pulp inflammation. In these animal models, however, interpretation of the results is difficult because bacteria were identified in the pulp using only non-specific morphological criteria. It was later demonstrated that the dental pulp could be colonised by blood-borne bacteria in the absence of previous inflammation. Ten guinea pigs were inoculated intraperitoneally with *C. burnetii*, a strict intracellular bacterium responsible for Q fever that is not part of the normal flora of guinea pigs. At 20 days post-infection, in two out of four animals, the dental pulp was positive using PCR targeting two specific molecular fragments: primers CB1/CB2 targeting the gene encoding superoxide dismutase (*sod*) and primers Trans1/Trans2 targeting the insertion sequence IS111. Positive PCRs were found in 20–50% animals depending on the molecular target (Aboudharam et al. 2000). In this model, blood cultures were positive until the 5th day post-inoculation and spleen cultures were positive until the 10th day post-inoculation. *C. burnetii* DNA was not detected in the dental pulp until day 15 post-inoculation. These data showed that it was possible to detect specific DNA sequences in the dental pulp of bacteraemic animals. Moreover, detection was possible even after the bacteraemia ended. Further studies demonstrated that infection could also be demonstrated by direct culture of pulp tissue (Aboudharam et al. 2004a).

11.3.2 Naturally Infected Specimens

11.3.2.1 Animals

11.3.2.1.1 Cats and Bartonella

The domestic cat (*Felis silvestris catus*) is a reservoir for *Bartonella henselae*, *Bartonella clarridgeiae*, and *Bartonella koehlerae* (Droz et al. 1999; Kordick et al. 1997; Regnery et al. 1992). These species can cause human disease after a bite or scratch from cats or a cat flea (*Ctenocephalides felis*) bite (Chomel et al. 1996; Jacomo et al. 2002). In cats, *Bartonella* species cause chronic bacteraemia, which might persist for over a year without clinical or haematological changes (Abbot et al. 1997; Chomel et al. 2003; Koehler et al. 1994; Kordick et al. 1995). The prevalence of *B. henselae* bacteraemia in cats has varied from 4% to 68% in studies conducted in various countries worldwide (Koehler et al. 1994; Boulouis et al.

2005; Cholmel et al. 1995, 1999; Heller et al. 1997). The chronic asymptomatic bacteraemia that occurs with *Bartonella* spp. is an exceptional event in mammals that can help us to study the age and evolution of the relationships between cats and *Bartonella* spp. To develop a protocol for examining the remains of ancient cats, we investigated methods for the molecular detection of *Bartonella* spp. in 11 stray cats that had been buried for a year. We found that dental pulp was a suitable tissue for the molecular detection of *Bartonella* spp. and that dental pulp from the canine teeth of cats was statistically more likely to be positive by PCR detection than other teeth (Aboudharam et al. 2005). In a further study, we demonstrated that the dental pulp from 3 of 19 domestic cats dating from the thirteenth to the sixteenth centuries contained DNA specific to *B. henselae* (La et al. 2004). Also, DNA of *B. quintana* was found for the first time in contemporary domestic cats from Marseilles (La et al. 2005) even though humans were the only previously known reservoir for this bacterium (Maurin and Raoult 1996). This study led us to propose that cats might be an emerging source of human infections with *B. quintana*, in agreement with the epidemiological data in some cases of human *B. quintana* infections.

11.3.2.2 Humans

11.3.2.2.1 Humans and Human Immunodeficiency Virus

The first report of human immunodeficiency virus (HIV) in dental pulp was made by Glick, who used PCR to show HIV in a maxillary central incisor from a seropositive patient (Glick et al. 1989). The authors suggested that other viruses, such as hepatitis B, might also reside in the dental pulp, that instruments used for root canal therapy should be handled with the same caution as other sharp instruments, and that dental pulp should be disposed of in accordance with guidelines for other infected tissues. A more systematic study using PCR showed HIV in 11/12 pulps extracted from the teeth of 12 HIV seropositive patients. In situ hybridisation provided the first demonstration that HIV infects fibroblasts in the dental pulp. Histology did not reveal inflammation in the pulp but the authors suggested that dental pulp fibroblasts act as a reservoir for HIV in the body (Glick et al. 1991).

11.3.2.2.2 Humans and Herpes Simplex Virus

Herpes simplex virus (HSV) infects the oral cavity and migrates along the trigeminal nerve, part of which innervates the dental pulp. In a study of 46 patients, 19/23 of whom were seropositive (Heling et al. 2001), DNA of HSV could not be detected by PCR in dental pulp (11 normal and 17 necrotic), saliva or periapical tissue. The authors concluded that there is insufficient HSV for PCR detection, or that the virus did not enter the dental pulp. The authors did not specify how many seropositive patients were tested by PCR. Saliva from all the seropositive patients was tested by PCR and found negative although 7.4% of asymptomatic patients have viable HSV in oral rinse specimens of saliva (Spruance 1984).

11.3.2.2.3 Humans and Prion Protein

Based on animal model studies, Blanquet-Grossard et al. (2000) aimed to detect prions in the dental pulp of eight Creutzfeldt-Jakob patients using Western-blot analysis with monoclonal antibody 3F4, based on the results obtained in an animal model (Ingrosso et al. 1999). Although prions were found in the brains of patients, they were not detected in their dental pulp. The authors, however, had reservations about their results and recommended caution in health workers dealing with dental problems in patients with Creutzfeld-Jacob disease.

11.3.2.2.4 Humans and *Yersinia pestis*

The first demonstration of *Y. pestis* DNA in human dental pulp was made using 400-year-old samples (Drancourt et al. 1998). The results were confirmed using two different molecular targets (*pla* and *rpoB* genes) for *Y. pestis*. This study was also the first to provide nucleic-acid-based evidence of septicaemia in ancient remains in which there were no bone lesions indicative of the condition. Similar studies would be useful in resolving the etiology of other historical outbreaks, and the approach could be generally applied to research in palaeomicrobiology. In a further study we found DNA of *Y. pestis* in the dental pulp of victims of the medieval Black Death using a "suicide PCR" protocol. Here, the primers are used only once, there is no positive control, and positive specimens are sequenced and confirmed by sequencing a second target. This specific protocol minimises the risk of vertical contamination. Evidence that this technique was successful was our finding of original gene sequences that differed from sequences of modern strains; this protocol was proposed as the standard PCR technique of choice to completely avoid contamination of materials with previously amplified sequences (Raoult et al. 2000). Our results enabled us to resolve the long dispute over the aetiology of the Black Death by showing that the disease was in fact plague caused by *Y. pestis*. Independently, a German research group also detected *Y. pestis* DNA sequences in the teeth of two individuals buried in the second half of the sixth century A.D., which supported the evidence for the presence of *Y. pestis* in the first recorded pandemic (Weichmann and Grupe 2005). In this study, however, the DNA template was extracted from teeth powdered after several decontamination methods. In another study (Pusch et al. 2004), Yersinial F1 antigen was found in skeletons of victims of the Black Death, again validating the above result. However, an English research group (Gilbert et al. 2004) tried to detect *Y. pestis* in samples of specimens dating from the period of the Black Death using different molecular targets. This study did not find any specific fragment of *Y. pestis* DNA, and found DNA of a *Yersinia* strain only using a 16S fragment.

The new approach of genotyping bacteria from ancient specimens has been proposed as an appropriate method to study the epidemiology of ancient outbreaks of disease. Indeed, *Y. pestis* is subdivided into three major biovars that have been proposed to be responsible for the three specific plague pandemics. We collected

dental pulp from individuals dating back to each of the two historical pandemics to test this hypothesis using the technique of multiple spacers typing (MST), which was developed in our laboratory (Drancourt et al. 2004). The results confirmed *Y. pestis* as the cause of the pandemics, and showed that the three pandemics were associated with the Orientalis biovar only (Drancourt et al. 2004).

11.3.2.2.5 Humans and *Salmonella enterica* serovar Typhi

The cause of the Plague of Athens has long been debated by scientists who have attempted to interpret Thucydides' descriptions of the disease. In 2006, dental pulp from remains in a mass burial pit dating from the outbreak (around 430 B.C.) was used to determine the probable cause of the Plague of Athens (Papagrigorakes et al. 2006). Although tests were performed, in random order, for several putative pathogens (*Yersinia pestis*, *Rickettsia prowazekii*, *Bacillus anthracis*, *Mycobacterium tuberculosis*, cowpox virus, *Bartonella henselae* and *Salmonella enterica* serovar Typhi) using previously developed protocols (Aboudharam et al. 2000, 2005; Drancourt et al. 1998, 2004; La et al. 2004) until a positive result was obtained, only DNA of *Salmonella enterica* serovar Typhi, using two molecular targets (*osmC*, *clyA* and *NarG*), was found in 3/3 of the teeth tested. In this study, the authors used dental pulp as the material of choice for retrospective diagnosis of bacteraemic agents and did not use dental powder (including dentine) since only dental pulp assures good vascularisation and durability, and is naturally protected from external contamination. The protocol enables dental pulp that has been protected inside teeth for centuries to be used as the only available equivalent to a blood sample in the diagnosis of an infection. Extreme measures were taken to prevent any possibility of exogenous contamination, including suicide PCR, targeting different genomic regions and blinded manipulations by different operators in several laboratories. The positive results revealed original sequences that were repeatedly obtained by independent operators, thus validating the results. Once again, dental pulp proved useful in providing clear evidence leading to retrospective diagnoses from ancient remains and helped determine the cause of the Antique Plague of Athens.

11.3.2.2.6 Humans and *Bartonella quintana*

B. quintana is the etiological agent of trench fever, which occurred in soldiers during World Wars I and II (Byam et al. 1919; Kostrzewski 1949). This bacterium has now also been reported to cause chronic bacteraemia and endocarditis in homeless and alcoholic patients in modern cities in both Europe and the United States (Drancourt et al. 1995; Brouqui et al. 1999; Spach et al. 1995; Stein and Raoult 1995) and bacillary angiomatosis in both HIV-infected and immunocompromised patients (Koehler et al. 1997). Using PCR and sequencing, *B. quintana* DNA has been detected in the dental pulp of a patient who had been successfully treated with antibiotics for *B. quintana* septicaemia 6 months previously (Aboudharam et al. 2004b). Blood

cultures were negative at the time the dental pulp was found positive, therefore suggesting that dental pulp was a sanctuary for *B. quintana* DNA. We have also shown that *B. quintana* can be detected in dental pulp from the remains of a person who died 4,000 years ago; this study was the first to demonstrate *B. quintana* DNA in ancient human remains (Drancourt et al. 2005). A recent study of dental pulp from the remains of Napoleon's soldiers showed that louse-borne infectious diseases caused by *B. quintana* and *Rickettsia prowazekii* affected nearly one-third of Napoleon's soldiers buried in Vilnius and might have been a major factor in the French retreat from Russia (Raoult et al. 2006). This study once again showed that dental pulp can be used for the retrospective diagnosis of infectious diseases.

11.3.2.2.7 Humans and *Rickettsia prowazekii*

Rickettsia prowazekii causes epidemic typhus in people during wartime and has been classified on the B list of potential bioterrorism agents by the Centers for Disease Control and Prevention (Atlanta, GA). The bacterium was found in the dental pulp of three soldiers from Napoleon's army (Raoult et al. 2006) and, since epidemic typhus results in high mortality, it was thought likely that the soldiers died of the disease. This study again confirms that searching for the DNA of infectious agent in dental pulp is an important tool in investigating the history of infectious diseases (Raoult et al. 2006).

11.4 Protocols for Molecular Detection of Microorganisms in Dental Pulp

11.4.1 Selection and Preparation of the Teeth

The selection of teeth is the first step (Box 11.1). To be suitable, a tooth must be intact, with a closed apex that will prevent external contamination – the most

Box 11.1 Suggested guidelines for the selection of teeth prior to total DNA extraction from dental pulp

For microbial detection, teeth should be transported and stored in separate containers at room temperature with the following stipulations:

Teeth should be intact with a closed apex

Teeth with single roots are preferred

Unerupted teeth should be tested immediately after being exposed to the external environment

More than one tooth per individual should be used if possible

important factor when working with ancient specimens. The presence of a closed apex should be reconfirmed after the tooth has been washed and subjected to external decontamination as it is sometimes very difficult to observe tiny defects because of the colour and nature of ancient specimens. In our experience, teeth with a single root are more suitable for study because they have a large volume of pulp and are easier to manipulate than teeth with multiple roots. The canine tooth of people and cats is particularly suitable because it has the largest volume of pulp. Unerupted teeth are totally protected in the jawbone but their apex is almost always open. Therefore, to prevent contamination, any experiment on unerupted teeth should be performed immediately after removing the tooth. The quality of ancient samples cannot be assured in all specimens, and, if possible, multiple teeth should be examined per individual. Once a tooth has been selected, digital photographs and radiographs are taken for identification purposes, and to record information on the specimens such as their form and colour. Radiographs enable operators to estimate the pulp volume, the presence of calcification in the pulp and the status of the apex. This is important as calcification of pulp and very small pulp volume, although rarely encountered, prevent the recovery of sufficient pulp material for proper detection of microorganisms. Our experience with a large collection of human teeth has indicated that the presence of dental pulp calcification in teeth makes the pulp unsuitable for nucleic acid extraction. Digital information (photographs and X-ray radiographs) is also useful for subsequent anthropological studies because the tooth suffers considerable damage when dental pulp is extracted. However, we have been able to reassemble and glue teeth back together after dental pulp removal and thus restore some of their initial appearance.

11.4.2 Removal of Dental Pulp

Dental pulp can be removed using different methods depending on its status and the purpose of the study (Figs. 11.4, 11.5, Box 11.2). Generally, teeth are extracted and decontaminated by wiping their external surface with bleach and by exposure to ultraviolet (UV) light before the dental pulp is removed. In one method, the entire tooth is crushed into a fine powder, decalcified, and nucleic acids then extracted. In a second method, which is applied to teeth from living people, the tooth is cleansed, isolated with a rubber dam, opened with a sterile bur and the dental pulp removed with a sterile broach (Glick et al. 1989, 1991; Heling et al. 2001). This method is rarely appropriate because we always try to keep dental pulp as alive as possible in the patient. This protocol should be used only in cases of irreversible pulpitis or other therapeutic indication for dental pulp removal. A third method consists of opening the teeth from ancient remains with a longitudinal fracture using a rotative disk, and scraping the dental pulp and its powdery remnants into a sterile tube for DNA extraction (Aboudharam et al. 2004a, 2005; Drancourt et al. 1998, 2004; La et al. 2004; Papagrigorakis et al. 2006; Raoult et al. 2006). This method is simple to carry out. All the above methods carry some risk of contamination because

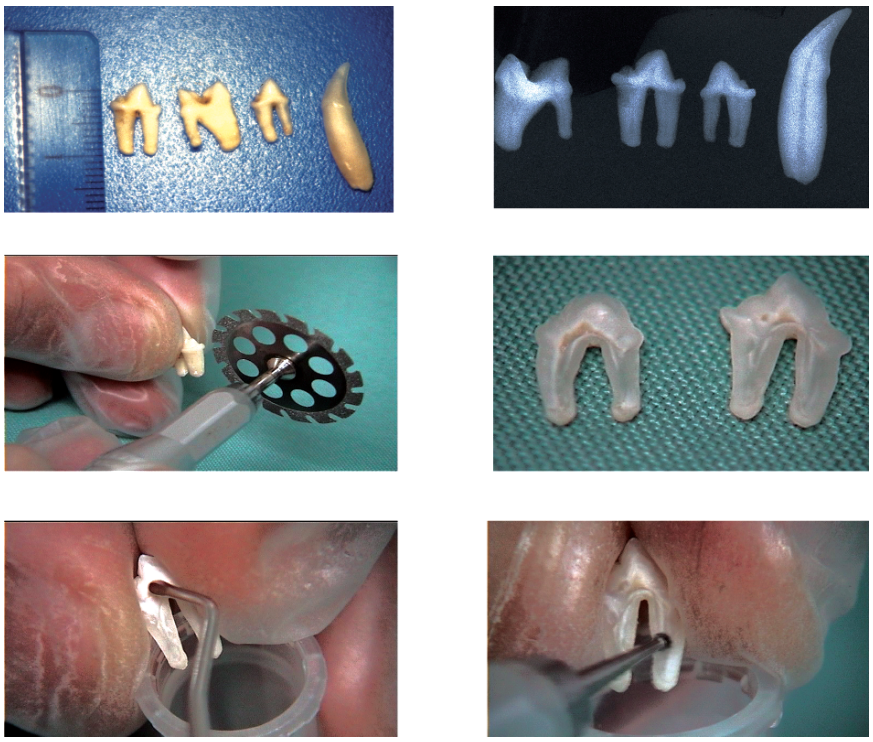

Fig. 11.4 Protocol for recovery of dental pulp from feline teeth

dental pulp is exposed to the environment while being removed. An original protocol for recovery of dental pulp via the apex was established by Gilbert et al. (2003). Here, the tooth is fully encased in silicone rubber, the top of the root is removed horizontally and the dental pulp is powdered and removed from the pulp chamber using a dental drill bit. In our experience, however, the silicone does not tightly adhere to the tooth; we tried to improve the protocol by firstly decontaminating the tooth with 70% ethanol and then placing the entire tooth in sterile resin (Resin Polyester SODY 33, ESCIL, Chassieu, France) poured into a sterile centrifugation tube (Millipore, Bedford, MA). After polymerisation of the resin at room temperature for 30 min, the apex of the root is removed using a sterile disk and the opened tooth, still embedded in the resin, is inserted upside down in a sterile Eppendorf tube and centrifuged at 8,000 rpm for 10 min to recover the dental pulp (La et al. 2005). This method has been applied to the recovery of dental pulp from contemporary teeth but not yet from ancient teeth. In ancient teeth, the pulpal chamber is not entirely occupied by dried dental pulp remnants, thus DNA extraction reagents can be injected directly into these cavities via the apex. This protocol uses the tooth itself as a sterile tube to contain the reagents but it requires a large pulp chamber.

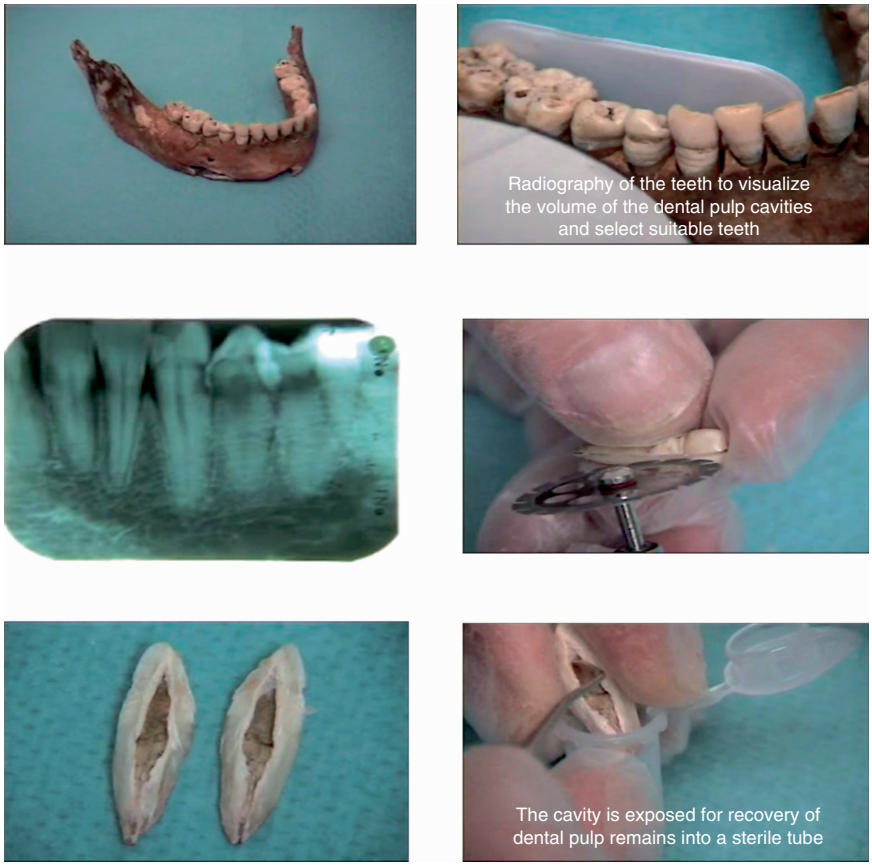

Fig. 11.5 Protocol for recovery of dental pulp from human teeth

Box 11.2 Suggested protocol for the recovery of dental pulp

Photography
Radiography
External decontamination
* 70% Ethanol
* Ultra violet (for immunohistology)
Enclose tooth with sterile resin using a type centrifugation tube
Moving top of the tooth to open root canal widely enough
With contemporary teeth, centrifuge the pulp into a collection tube (10 min at 8,000 rpm)
With ancient teeth, inject extraction reagents (Proteinase K, SDS) and incubate according to extraction protocol prior to the above step

For that reason, continued dentine formation results in a reduced volume of the dental pulp chamber, which precludes the use of this method (L. Tran-Hung, personal communication).

11.4.3 Extraction of Nucleic Acids

Dental pulp is a soft tissue and any extraction protocol can be used to extract nucleic acids directly without a decalcification step. In our experience with ancient specimens, the phenol–chloroform protocol for total nucleic acid extraction is the optimal protocol. Controls are essential to detect contamination but, to avoid the risk of contamination, positive controls should not be used.

11.4.4 Genomic Amplification

We select molecular targets of under 300 bp, carry out amplification reactions in small volumes (final volume: 25 µl), and add BSA (bovine serum albumin) to our reaction mixture to reduce the influence of inhibitory factors present in ancient samples. We have not used positive controls in our PCR-based experiments, preferring to use a "suicide PCR" protocol to minimise the risk of contamination.

11.4.5 Prevention of Contamination

This is the most important aspect of the procedure and, depending on the nature of the sample, we use a variety of methods for external decontamination that include cleaning teeth with distilled water, immersing them in absolute or 70% ethanol, and/or exposing them to UV light. The best protocol is to totally cover the tooth with sterile resin and then recover dental pulp through the apex by centrifugation. This technique, however, is not suitable for molars with very narrow root canals. It is most useful for teeth with single roots.

Every step in the experiment should be performed in a separate room with disposable equipment and newly prepared reagents. All PCR-based experiments should be carried out in designated one-way PCR suites with appropriate ventilation. "Suicide PCR" reactions that target a new genomic region may prevent vertical contamination from previous amplifications. The introduction of numerous contamination and negative controls in any amplification reaction may help to ascertain the source of any contamination. In our laboratory, we now use one negative control and one contamination control for every four ancient samples we process.

11.5 Applications in Palaeomicrobiology

Dental pulp has been used for the detection, identification and characterisation of microorganisms in ancient remains using PCR-based molecular techniques (Drancourt et al. 1998, 2004, 2005; La et al. 2004; Papagrigorakis et al. 2006; Raoult et al. 2000, 2006; Weichmann and Grupe 2005).The consecutive publication of several papers implementing the more or less same experimental protocol underlies the increasing worldwide acceptance of its validity and applicability on several relevant matters of historical and medical interest. Thus, investigating dental pulp has allowed us to suggest certain bacteria as the possible etiologic causes involved in the host's bacteraemia, and has helped interpret historical and anthropologic data in which there was no microbial evidence and where it is hard to find exact descriptions in comparison to modern pathology due to ancient language and problems of translation. These studies strongly encourage other researchers who are interested in investigating the history of infectious diseases to use dental pulp as the material of choice for their research. Recently, by using dental pulp, we clearly demonstrated that louse-borne infectious diseases affected nearly one-third of Napoleon's soldiers buried in Vulnius, and indicated that these diseases might have been a major factor in the French retreat from Russia (Raoult et al. 2006). Similarly, a Greek team led by Manolis Papagrigorakis successfully applied this technique to shed light on one of the most debated enigmas in medical history, the cause of the Plague of Athens (Papagrigorakis et al. 2006); this report pinpoints typhoid fever as the disease responsible for this devastating epidemic. Hence, investigation of dental pulp allows us to diagnose past infectious diseases and elucidate past epidemiologies by determining the causative organism. However, a fundamental problem is the need for careful measures to protect material from external contamination. In order to validate the data, we proposed a set of criteria that can be used to assess results obtained from ancient specimens (Box 11.3). Because of its durability, using dental pulp to detect microorganisms might help us determine the time during which the microbes infected the host. Dental pulp can also be used to provide information on emerging infectious diseases by helping to establish models of emerging infections and by contributing to the development of appropriate preventive measures (Drancourt and Raoult 2005). In fact, we unexpectedly discovered that cats could be infected by presumably bacteraemic *B. quintana* (La et al. 2005). This observation is very useful in understanding the natural epidemiologic cycle of *B. quintana*, from which we can recommend that immunocompromised patients avoid contacts with cats. For the first time, we were able to demonstrate this missing link in *B. quintana* infections by using dental pulp. Therefore, we can apply this approach to identify *Bartonella* spp. in other animals and locations, especially in ancient animals; this will help in the understanding of the geographical distribution of this bacterial genus. We have been able to show that the co-evolution between *B. henselae* genotype Houston and cats existed at least 800 years ago in France, even though the first description of cat scratch disease (CSD) caused by *B. henselae* dates from 1950 (Debré et al. 1950). The high prevalence of *Bartonella* spp.

Box 11.3 Criteria for the authentication of molecular data in palaeomicrobiology (Drancourt and Raoult 2005) (reproduced with permission from Nature Reviews)

Absence of a positive control
The positive control should be removed from the laboratory in which ancient specimens are processed
Negativity of negative controls
Several negative controls should be analysed in parallel with the specimens being processed
Negative controls should be as similar as possible to the ancient specimens
Negative controls should remain free of amplicons
Sequencing of PCR amplicons
PCR alone does not ensure the specificity of the diagnosis, and amplicons have to be sequenced to identify ancient microorganisms
Targetting a new sequence in the laboratory
PCR should target a specific sequence that has not previously been amplified in the laboratory
Amplification and sequencing of a second target
A positive result must be confirmed by amplification and sequencing of a second specific molecular target
Originality of the ancient sequence
Acquisition of an original sequence that differs from modern homologues by mutation or deletion excludes contamination

bacteraemia in cats supports the possibilities of frequent exposure, persistent infection, and recurrent infection with this bacterium in cats. Therefore, it can be assumed that cats have long been infected by this bacterium and that the two have co-evolved over centuries. This may provide very reasonable explanations of some historical medical sources, such as reports of the French and English kings' apparent power to cure scrofula in medieval times – some of the cases may have been self-limiting CSD (La et al. 2004). **T**he antiquity of this co-evolution, in which cats and *B. henselae* have interacted over many centuries, could be further studied by increasing the number of specimens as well as the time frame they cover. It would also be very interesting to enlarge the number of ancient cat samples from different countries, such as those of the New World, in order to study the genomic variations and the origin of this bacterium. Indeed, this seems very feasible because we have already demonstrated that dental pulp is useful not only for detection of infectious

diseases but also for genotyping ancient bacteria (cf. our *Y. pestis* study, see above) and such studies have the potential to contribute greatly to genomic research (Drancourt et al. 2004; Drancourt and Raoult 2005). We recommend that dental pulp of ancient remains be used for retrospective diagnoses; pathogens found in dental pulp might have been associated with bacteraemia in the host. We would note that viable bacteria can be found in dental pulp even though blood cultures are negative or unavailable (Aboudharam 2004a, 2004b). Therefore, we can use dental pulp to search for blood-borne microorganisms in cases where blood tests are not possible, as in ancient specimens and, in certain cases, investigation of dental pulp may provide evidence to support putative historical hypotheses (Papagrigorakis et al 2006; Drancourt and Raoult 2005). Because targeting a specific pathogen has its limitations in molecular diagnosis, the 16S rRNA gene has been used as a universal detection fragment for bacteria. However, this approach is prone to problems of contamination. The only attempted 16S rRNA gene-based detection of bacteria in ancient dental pulp to date resulted in contaminated amplifications. Therefore, a more universally applicable protocol will be required to promote the use of dental pulp in palaeomicrobiology.

11.6 Conclusions

Bacteria can colonise dental pulp via the haematogenous route, and their presence can be demonstrated in this tissue by using molecular techniques in both contemporary and ancient specimens, as well as in culture-based experiments for contemporary specimens. Further investigations are required to determine the range of bacteria that can colonise dental pulp from the blood. In some infections, dental pulp is considered as a sanctuary for microorganisms, which can be detected by PCR even after blood cultures become negative. This might be because these microorganisms are no longer in the bloodstream but can reside in the dental pulp; this tissue can then be used to detect blood-borne pathogens even if blood cultures are not available or are found to be negative. A summary of published data (Table 11.1) shows that our laboratory has contributed greatly to this field of research since 1998 by developing and improving the techniques required. From our experiences and the available literature we would note that there is an increased chance of finding bacteria in teeth that have a large volume of dental pulp, e.g. the canine teeth of cats and humans, as compared to other teeth. It is easier to obtain sequences using contemporary teeth, as positive samples can be sequenced directly. With ancient teeth, however, molecular cloning is usually necessary to determine sequences from positive samples. Prevention of contamination is essential, as ancient samples are unique and there are few available materials to use for further investigations.

Table 11.1 Summary of published studies of detection of infectious agents in dental pulp. *PCR* Polymerase chain reaction, *HIV* human immunodeficiency virus, *HSV* herpes simplex virus

Source	Date	Infection method	Method	Number of tested teeth	PCR target	Microorganism	Reference
Dog	Modern	Intravenous with irritation	Culture / histology / immunohistology	109		*Escherichia coli* beta-hemolytic *Streptococcus*	Aboudharam et al. 2004a
Dog	Modern	Intravenous with irritation	Histology	36		*Streptococcus* spp.	Aboudharam et al. 2004b
Cat	Thirteenth–sixteenth century	Natural colonisation	PCR	135	*groEL*; *Pap31*	*Bartonella henselae*	Papagrigorakis et al. 2006[a]
Cat	Modern	Natural colonisation	PCR	9	*ITS*; *Pap31*	*Bartonella henselae*; *Bartonella quintana*	Raoult et al. 2000[a]
Cat	Mimic ancient	Natural colonisation	PCR	104	*groEL*	*Bartonella henselae*; *Bartonella* spp.	Drancourt et al. 2004[a]
Guinea-pig	Modern	Intraperitoneal without irritation	PCR	280	*Sod*; *IS111*	*Coxiella burnetii*	Regnery et al. 1992[a]
Guinea-pig	Modern	Intraperitoneal without irritation	Culture	52		*Coxiella burnetii*	Aboudharam et al. 2005[a]
Human	Modern	Natural colonisation	Culture / histology	30		*Streptococci*	Tziafas 1989
Human	Modern	Natural colonisation	PCR	1	Antibodies to HIV	HIV	La et al. 2005
Human	Modern	Natural colonisation	PCR / hybridisation	12	Antibodies to HIV	HIV	Blanquet-Grossard et al. 2000
Human	Modern	Natural colonisation	Western blot	8		Prion protein	Gilbert et al. 2004
Human	1348 A.D.	Natural colonisation	PCR	23	*pla*	*Yersinia pestis*	Ricaut et al. 2005[a]
Human	Sixteenth–eighteenth century	Natural colonisation	PCR	12	*pla*; *rpoB*	*Yersinia pestis*	Drancourt et al. 2005[a]

Human	Fifth–fourteenth century	Natural colonisation	PCR	19	Multiple intergenic spacers	*Yersinia pestis*	Glick et al. 1989[a]
Human	Modern	Natural colonisation	PCR	1	*groEL*; *hppE*	*Bartonella quintana*	Drancourt et al. 1998[a]
Human	2000 B.C.	Natural colonisation	PCR	12	*groEL*; *hppE*	*Bartonella quintana*	La et al. 2004[a]
Human	Sixth century	Natural colonisation	PCR	2	*pla*	*Yersinia pestis*	Delivanis et al. 1981
Human	Modern	Natural colonisation	PCR	28	Quanti-PATH HSV 1,2 KIT	Herpes simplex virus	Ingrosso et al. 1999
Human	Thirteenth–seventeenth century	Natural colonisation	PCR	108	16S; *pla*	*Yersinia pestis*	Aboudharam et al. 2000
Human	Eighteenth century	Natural colonisation	PCR	47	*glpD*	*Yersinia pestis*	Drancourt et al. 2007[a]
Human	1812	Natural colonisation	PCR	86	16S; *pla*	*Bartonella quintana*; *Rickettsia prowazekii*	Gilbert et al. 2003[a]
Human	430 B.C.	Natural colonisation	PCR	3	*OsmC Et clyA* and *narG*	*Salmonella entica* (serovar *Typhi*)	Wiechmann and Grupe 2005
Human	Modern	Natural colonisation	PCR	51	16S; and *rpoB*	All bacteria	L. Tran-Hung et al. 2007

[a] Studies performed in our laboratory - 13/23 (56 %)

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