

Endodontic Microbiology

Endodontic Microbiology

Second Edition

Edited by

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Dedication

To Amal, Fikry, Lori, Amani, George, Anthony Gade, and Edward; thank you for providing me the opportunity, the inspiration, the motivation, and the love.

Ashraf F. Fouad

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Preface

Much has happened in endodontic microbiology since the publication of the first edition of this book. Hundreds of important research studies and reviews have been added to the literature in this important field. We now have many better epidemiologic studies on the prevalence of endodontic disease, its association with systemic disease, and its potential contributions to major morbidity and mortality of patients. The area of polymicrobial infections is now recognized as a major public health problem. In the last decade it has seen innovations in research methodologies as well as the conceptual descriptions of how these infections can produce disease. In the field of endodontic microbiology, next generation sequencing is now commonly used in research, revealing hundreds if not thousands of microbial taxa that are involved in endodontic pathosis. The study of microbial virulence has also seen major advances. These include the interplay of different pathogens, such as bacteria, viruses, and fungi, to increase the pathogenicity of either, the host–microbial interactions, the differences in clinical presentations, and responses to treatment observed with different genomic and epigenetic variations in the host, bacterial load issues, quorum sensing, and the keystone pathogen concept that describes how a pathogen can induce host changes that converts a microbial community to become dysbiotic.

Endodontic microbiology research still has many frontiers that have not been adequately studied. These include the reasons why chronic infections can exacerbate to produce severe and spreading infections, the degree to which endodontic microflora travel to distant

sites in acute and chronic infections, the exact relationship between residual bacteria and healing, and the effects of residual bacteria on the success of regenerative therapies. The interaction of the microbial community in the deep carious lesion or the necrotic pulp with the host response can produce chronic asymptomatic disease or severe pain. The degree to which the composition of the microflora, the expressed virulence factors, and the host's innate susceptibility to disease interact to produce the resultant clinical manifestation needs further elucidation.

The ability to eliminate microbial irritants is paramount to adequate healing in endodontics. There are still no clinical markers that can predict the long-term responses to vital pulp therapy or to endodontic treatment. Scientific explorations that utilize cutting edge technologies, such as shotgun sequencing or metagenomics, transcriptomics, and proteomics have not been sufficiently incorporated in endodontic research. The degree to which microbial elimination is required to mediate the regeneration of the dental pulp, or even just revitalization in the pulp space, is not clear. Finally, we still do not have rapid molecular methods of identifying antibiotic resistance, which would allow the efficient and effective selection of the right antibiotic. These and many other questions will continue to inspire many studies and insights that would allow us to improve the success of our treatment modalities, save more teeth from extraction, and improve the patients' quality of life.

Ashraf F. Fouad

Preface to the First Edition

Endodontic infections are very prevalent, because they mostly represent complications of dental caries and its treatment, as well as traumatic injuries to teeth, which are all very prevalent occurrences. Collectively, they represent the majority of dental infections that present with significantly acute local and systemic signs and symptoms. This is the first textbook devoted to the study of endodontic infections, which hitherto has been limited to isolated single chapters in endodontic textbooks. This textbook is intended to provide a collection of work showing the state of the knowledge in this field. It is also intended to provide some research questions and hypotheses that, hopefully, will stimulate more efforts to understand the disease process and identify effective treatment methods.

The study of endodontic microbiology has been complicated by difficulty in epidemiological data in obtaining adequate endodontic diagnosis on large numbers of nonpatient populations. In addition, sampling is a major challenge in endodontics. Contamination from the tooth surface, caries, or saliva must first be avoided. Access to the potentially very complex root canal anatomy and disruption of biofilm on the majority of canal walls in these areas is necessary. It is almost impossible to differentiate specimens obtained from the apical and coronal portions of the root canals; thus, the effect of location of microflora within the canal is poorly understood, and can only be studied in teeth that are extracted. Finally, sampling after completion of treatment to assess effectiveness of treatment and determine the long-term outcome risk is complicated by the fact that only the areas that could be reached could be sampled.

The difference in sensitivity between traditional culturing and modern molecular methods are

especially important in endodontic microbiology, because the endodontic specimen has so little material, and sensitivity, therefore, has a major role in microbial identification. The description of traditional bacterial pathogens and their virulence factors represents most of the available literature today. The contributions of the not-yet-cultivated bacteria and the bacteria rendered temporarily uncultivable by traditional treatment methods have not been adequately studied. Likewise, we are just beginning to understand some of the contributions of fungi and viruses to the pathogenesis of endodontic infections.

The debate on viable versus dead microorganisms that are detected by molecular techniques must be resolved by using more accurate technologies that assess microbial counts, their viability, and their pathogenicity. Likewise, consistent and stringent methodologies, including sequencing of amplification products, are essential for assuring accurate results and enabling comparisons among studies.

Persistent endodontic pathosis may be due to persistent infection or new infection after treatment. Sampling of apical lesions during periapical surgery is complicated by the lack of sterility of the surgical field. Therefore, the microbiology of nonhealing endodontic cases is still in its infancy at this time.

It is clear that in order to determine effective treatment modalities, better sampling and identification techniques must be employed and more adequately designed outcome studies need to be performed.

Finally, the relationship between endodontic pathosis and systemic disease must be more comprehensively studied. Endodontic infections were historically thought to contribute to numerous systemic diseases. While the potential for systemic spread of an acute

endodontic infection is well-known and documented, earlier studies have failed to demonstrate that chronic endodontic infections contribute to systemic diseases. However, these hypotheses must be reexamined now that we have more accurate research tools. In addition, the creation of large patient databases for longitudinal

analysis of treatment outcomes, and their relationships with systemic disease will be imperative in future studies that address this issue.

Ashraf F. Fouad

Chapter 1

Microbial Perspectives in the Twenty-First Century

William Wade

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| 1.1 Introduction | 1.6 Bacterial–bacterial communication |
| 1.2 Genomics | 1.7 Host–bacterial interactions |
| 1.3 Molecular microbial ecology and the study of uncultivable bacteria | 1.8 Complex infectious diseases |
| 1.4 Intraspecies variation | 1.9 The future |
| 1.5 Metagenomics and metatranscriptomics | 1.10 References |

1.1 Introduction

The final quarter of the nineteenth century was arguably the golden age of medical microbiology. The ground-breaking work of Pasteur, Koch, and others led to the development of broth and agar media that were able to support the growth in the laboratory of the major bacterial pathogens affecting humans. The ability to grow these organisms in pure culture led to the production of vaccines for many of the diseases they caused. These advances, and the subsequent discovery and development of antimicrobials, led to the mistaken belief that infectious disease had been beaten.

Of course, it is now realized that this optimistic viewpoint is not justified, not least because of the rapid emergence of bacterial resistance to antimicrobials. Indeed, the consensus view is that the battle against bacterial resistance is currently being lost, because of both the difficulty and costs associated with developing new antimicrobials and indiscriminate use of those currently available. The predicted ultimate failure of antimicrobial strategies has led to renewed interest in elucidating the pathogenic mechanisms used by

bacteria to cause disease, with the ultimate aim of devising new methods of disease prevention and treatment.

At the same time, interest in the microbial populations of the Earth has been intense and new techniques have become available to characterize the bacterial communities found in every ecosystem on the planet. These have revealed the quite astonishing diversity of microbial life on Earth and the extreme complexity of most bacterial communities. Furthermore, the extent of subspecific diversity is only now being fully appreciated. Bacteria readily exchange DNA and can “shuffle” their own genomes to generate diversity with the ultimate aim of responding and adapting to environmental change. As discussed later, bacteria in communities communicate with each other and, in the case of commensals living with plants and animals, their hosts. These interactions operate at various levels and can be remarkably sophisticated. The twenty-first century will be a period of tremendous advances in our understanding of the microbial world.

The aim of this chapter is to review recent developments in microbiology and to highlight selected

areas that are likely to change our conceptual view of infectious disease as a whole, and oral and endodontic infections in particular. Inevitably, a single short chapter cannot provide a comprehensive overview of an entire discipline, but the interlinked topics covered are those that will undoubtedly change our view of the microbial world and its relationship with the human host.

1.2 Genomics

The sequence of the human genome was published in 2001. The benefits of this outstanding achievement are now being realized with the identification of genes responsible for or causing a predisposition for a large number of diseases (Wellcome Trust Case Control Consortium 2007). At the same time, and largely possible because of the technical advances made as part of the human genome sequencing effort, genomes of other organisms are being sequenced, including those of bacteria.

As of February 2015, the sequencing of the genomes of 26 522 bacteria and 647 archaea had been completed, while 15 800 and 424, respectively, were in progress or available as a draft (for more information see www.genomesonline.org). As expected, the data obtained have revealed the enormous genetic potential contained within bacterial genomes; in each genome sequenced, around one-third of the genes present have been novel and the function of a significant proportion remains unknown.

The availability of genome sequence data is allowing a far more robust bacterial classification to be constructed than previously possible. Bacterial taxonomy was once based purely on phenotypic characters and was very inexact because of the difficulties involved in obtaining and interpreting such data compared to plants and animals where differences in phenotype are far more obvious. In recent years, genetic information has been increasingly used, but on a limited scale, and typically only the sequences of the 16S ribosomal RNA (rRNA) and other housekeeping genes have been used. New methods are now being introduced to make use of the sequence data available for complete genomes (Konstantinidis and Tiedje 2005). In general, the results of using such methods have supported the 16S rRNA gene taxonomy at species and genus level but, in addition, have provided improved clarity of the relationships among the higher taxonomic

ranks, where substantial overlap between ranks has been observed.

The results of the analysis of some genomic data have been extremely surprising. A Gram-positive coccus found in amoebae could not be identified by the conventional molecular analysis of 16S rRNA gene sequencing because no ribosomal genes could be amplified for sequencing. Genomic data explained this difficulty by revealing that the organism was actually a virus, the largest yet discovered. Now named *Mimivirus*, the large virus particles are up to 0.8 μm in diameter, the size of many bacteria. It primarily infects amoebae but has been implicated as a cause of pneumonia on serologic grounds and has caused a laboratory-acquired pneumonia in a researcher (Raoult et al. 2007). At the other end of the bacterial scale, members of the genus *Epulopiscium*, found in the intestine of certain surgeonfish (Angert et al. 1993), have been discovered that are visible with the naked eye.

In addition to correctly identifying evolutionary oddities, genomic data have identified numerous novel biochemical pathways with the potential for exploitation. Among these are some novel antimicrobials although the range of targets within bacterial cells that has arisen by natural evolution is rather narrow. A more promising avenue to the development of novel antimicrobials is to use genomic data to identify novel targets for antimicrobial treatments (Pucci 2006). Predictions can be made from genome data as to how essential a given gene is to an organism and therefore how disrupting the gene would affect the vitality of the organism. These predictions can then be tested in an appropriate manner experimentally using a wide range of methods that have been developed in response to the availability of genomic data. These include random mutagenesis mediated by transposons or insertion of plasmids, targeted gene disruption or in vivo techniques such as signature-tagged mutagenesis and in vivo expression technology. Structural genomics, where sequence data is used to predict the structure of essential bacterial proteins, is also being used to identify potential targets for antimicrobials. Finally, comparative genomics can be used to identify common features of pathogens affecting a particular body site to custom design antimicrobials for specific purposes, for example, respiratory tract infection.

Next generation sequencing technologies such as the Roche 454 and Illumina systems have been introduced and have brought the ability to sequence

bacterial genomes within the reach of individual laboratories. Accurate interpretation of the data remains a challenge, however, although a number of useful software programs are now available (Edwards and Holt 2013). The information obtained thus far has been of extraordinary value in understanding the role of pathogenic bacteria in disease and is the fundamental basis of other new technologies such as transcriptomics and proteomics. The next task will be to understand how gene products interact both within a bacterial cell and in response to external stimuli from the environment and other organisms.

1.3 Molecular microbial ecology and the study of uncultivable bacteria

Almost without exception, oral infections are polymicrobial in nature and difficult to study because around half of the bacteria present in the oral cavity cannot be grown using conventional culture media. It has long been recognized that not all bacteria from a given habitat can be cultured on artificial media in the laboratory. Indeed, it has been estimated that less than 2% of bacteria on Earth can be cultured.

Methods for the characterization of complex bacterial communities were developed as a consequence of the use of DNA sequence data for the construction of evolutionary trees. This was done by comparing the sequences of genes encoding essential functions, the so-called housekeeping genes that are found in all cellular organisms. The gene most commonly used to date has been encoding the small subunit (16S) rRNA molecule. Ribosomes have the essential function of translating messenger RNA (mRNA) into amino acid chains and, because of the need to preserve function, have evolved slowly. Some of the regions of the gene have changed very little over time and are therefore virtually identical in all bacteria. These regions are very useful for the design of *universal* polymerase chain reaction (PCR) primers that can amplify the gene from a wide range of different bacteria. Other regions are more variable and can be used to discriminate between organisms, almost to species level. Woese and colleagues used small subunit rRNA comparisons to construct a tree of life (Figure 1.1), which showed that bacteria had evolved into two domains, the Archaea and Bacteria, while eukaryotic organisms fell into a single third domain, the Eukarya (Woese 1987). It was

originally thought that organisms found in the domain Bacteria were those found in *normal* environments while the Archaea were present in extreme environments such as the deep sea and associated with volcanoes and so on. However, these associations have since been shown not to be true and members of Archaea are now known to be widely distributed, and an archaeal genus, *Methanobrevibacter*, can be found in the human mouth.

A major consequence of the availability of this tree is that unknown organisms of any type can be identified simply by sequencing their rRNA gene and adding the sequence to the tree or by directly comparing the sequence with the hundreds of thousands of bacterial sequences held in the sequence databases. Complex bacterial communities can be characterized by the PCR, cloning, and sequencing of 16S rRNA. Such studies have been performed with samples from the human mouth in health and disease and are described in more detail in Chapter 5 and throughout the textbook. A common finding of every study to date has been to confirm that around half of the oral microbiota is uncultivable. Around 700 species have been detected, 95% of which belong to the phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Fusobacteria, and Spirochaetes (Dewhirst et al. 2010). Other phyla consistently detected are Synergistetes, Chloroflexi, and the un-named phylum-level divisions GN02, SR1, and TM7 (Camanocha and Dewhirst 2014). The Human Oral Microbiome Database (www.homd.org) lists the bacterial taxa found in the mouth and provides descriptions of their phenotypes, where available, with links to genome sequence data as well as a 16S rRNA gene sequence identification tool (Chen et al. 2010).

Major efforts are now being made to improve our understanding of currently unculturable bacteria. These include the development of new culture media that better mimic the natural environment. Very often, laboratory culture media are far richer in nutrients than the natural habitat and the use of dilute media or filtered natural substrate has been successful in culturing previously uncultured organisms. This approach has not been applied systematically to the study of oral unculturable bacteria, but should be possible. Around half of oral bacteria cannot be cultivated *in vitro*. There appears to be no single reason for this but it has been shown that because oral bacteria naturally live in a multispecies community, some species require the presence of other bacteria to grow. The development

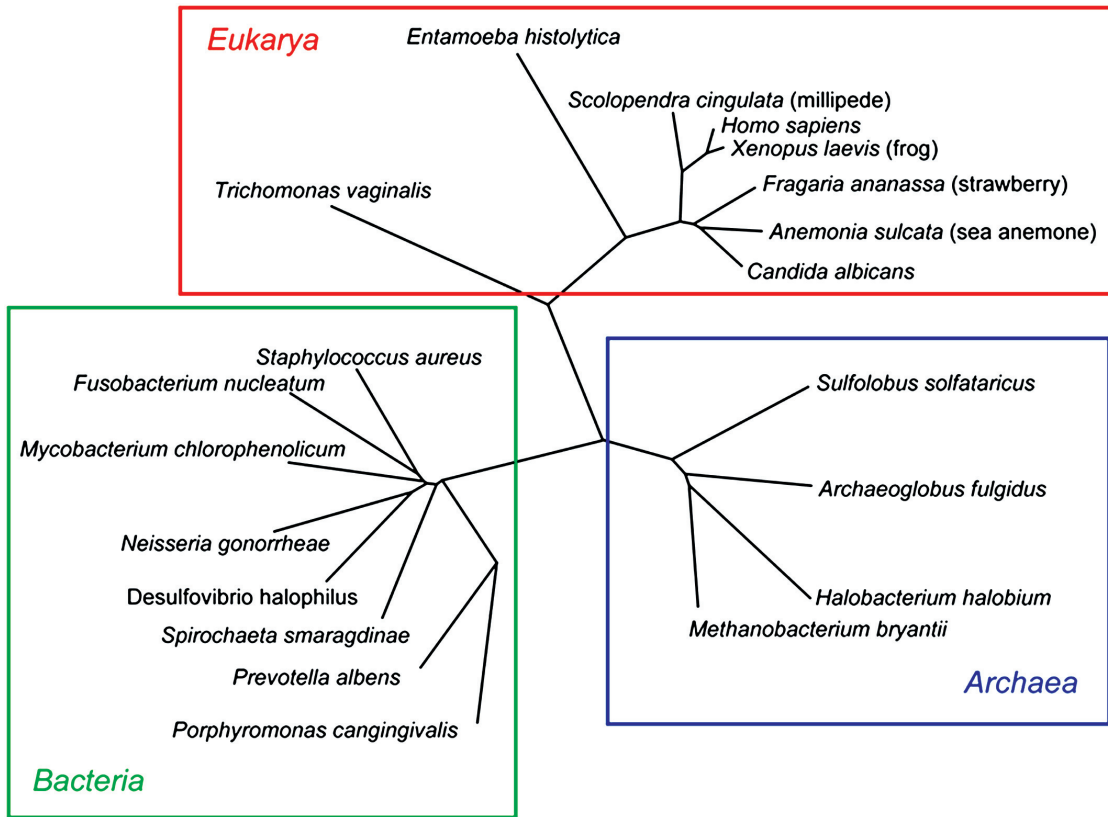


Fig. 1.1 Phylogenetic tree showing representatives of the domains Eukarya, Archaea, and Bacteria.

of coculture methods linked with enrichment of target organisms by in situ hybridization has enabled the cultivation of members of a previously uncultivated lineage of the phylum Synergistetes (Vartoukian et al. 2010). The commonly occurring division TM7 has long been a target for cultivation and it has been found that it can be detected readily in mixed laboratory cultures but not cultured independently (Hugenholtz et al. 2001). The reason for this observation has been determined recently in that a TM7 phylotype has been revealed to be an obligate episymbiont of other bacteria, and also capable of entering the host bacterial cells (He et al. 2015). It has a small genome (approximately 0.7 MB) and lacks genes for the synthesis of essential amino acids and so presumably needs to obtain amino acids from its associated bacterial host. TM7 is a large phylum widely distributed in the environment as well as being a component of mammalian microbiomes and it will be interesting to determine if all representatives

of the division are episymbionts or if some have other lifestyles.

The potential benefit of growing previously uncultivable organisms has been demonstrated recently in the successful attempts to cultivate soil bacteria by providing a natural community in contact with the culture system via a permeable membrane which has led to the discovery of a new antibiotic, teixobactin (Ling et al. 2015).

1.4 Intraspecies variation

The majority of microbiological diagnostic methods identify the target organism to species level. However, it is now recognized that individual strains within a species often vary markedly in their virulence. Within a species, some strains may be pathogenic while others are harmless. The extent of the genetic variation within

species, however, has only been fully realized by the sequencing of the genomes of multiple representatives of the same species.

In one such study, three strains of *Escherichia coli* were compared: the well-known harmless laboratory strain K12, an enterohaemorrhagic serotype O157 strain of the group associated with beef products, and a uropathogenic strain. It was found that they had only 39% of their genes in common, a surprisingly small number (Welch et al. 2002). These common genes encoded the functions that gave the strains their identity as members of the species *E. coli*, while the remaining genes gave them the ability to colonize particular body sites and/or damage the host by means of a specialized set of virulence factors appropriate for their natural habitat and lifestyle. Genes acquired from other organisms by horizontal gene transfer can be critical to that organism's behavior, and in the past may have been the reason a species was given a particular name. For example, if the mainly harmless environmental organism *Bacillus cereus* acquires plasmids pXO1 and pXO2, which carry genes coding for four toxins and the enzymes required to make a capsule, it becomes *Bacillus anthracis*, the causative agent of anthrax.

This work has given rise to some new genomic concepts. The core genome is that shared by all strains of the species, while the peripheral or accessory genome includes genes found in some strains but not others, but which nonetheless may be important in pathogenesis. Some bacteria go further and have two chromosomes; in this case, one normally encodes housekeeping genes and the second genes that confer fitness for competition in the environment.

The range of genes encoded by the peripheral genome can be extensive. In a study of the genome sequences of eight *Streptococcus agalactiae* strains, the authors calculated the number of strains of the species that would have to be sequenced to reveal the full genetic diversity of the species (Tettelin et al. 2005). The result was infinity. In other words, *S. agalactiae* can incorporate DNA into its genome from such a wide range of sources that all the possible genes that could be found within this species will never be known.

The implications of these findings are significant. Although much work has been invested in the development of rapid assays to detect the presence of specific organisms in clinical samples, including those collected from oral diseases, the association of a species

with disease may be insufficient for diagnostic purposes. Detection of the presence (and expression) of specific virulence genes may be required to provide a meaningful microbiologic diagnosis. This will clearly be difficult for diseases where the virulence determinants important in disease are currently unknown or where multiple virulence mechanisms are operating.

1.5 Metagenomics and metatranscriptomics

As individual bacterial strains can vary greatly in their genetic composition and the assignment of an isolate to a species alone is likely to give a poor indication of its pathogenicity, alternative methods of analysis need to be developed to determine the role of bacterial communities in human disease. New methods are particularly needed for complex diseases because the bacterial communities associated with the mucous membranes, where these diseases primarily occur, are so diverse that their routine characterization is not practicable. A novel approach does not attempt to isolate and purify all of the component species and strains, but, rather it considers the whole community and all of its constituent genes as a whole. The bacterial community found at a habitat is termed the *microbiome* and all the genetic material of the community members is the *metagenome* (Rondon et al. 2000).

The first stage in any such analysis is to extract DNA from all of the bacteria present in the sample. In early metagenomics studies, the DNA was cloned into either small plasmid vectors for ease of sequencing or into systems such as bacterial artificial chromosomes (BAC), which allowed DNA fragments up to 100 kb in length to be stably maintained in an *E. coli* host and genes expressed to seek functions of interest. These approaches have now been superseded by the use of next generation sequencing, which does not involve a cloning step. The cloning approach has allowed a number of new antibiotics to be discovered from metagenomic analyses of soil and marine environments. For example, turbomycin A and B were discovered in this way (Gillespie et al. 2002). Interestingly, a single gene was responsible for the activity which was mediated by an interaction between indole, normally produced by *E. coli*, and the gene product. The success rate in identifying novel antimicrobials in metagenomic libraries has been low; typically, several

hundred thousand clones have to be screened to find one new active compound. This relative lack of success partly reflects the methodology where *E. coli* is used as the host. Successful expression may require the presence of specific promoters or other accessory molecules and factors such as the G+C content of the insert and codon usage may adversely affect expression. Thus, in general, cloned fragments of DNA are expressed most easily in their natural host and the more phylogenetically distant the clone host, the less likely that expression will be successful. To overcome this, new vector–host systems are being introduced for the expression of metagenomic libraries to enable a better match between the insert and the host in which it is being expressed. These include *Streptomyces* and *Pseudomonas*, two genera members of which naturally produce secondary metabolites with properties of interest. An alternative approach is to use bioinformatic methods to screen for gene clusters encoding potentially useful compounds within metagenomic data. This approach has been successfully applied to environmental samples (Owen et al. 2013).

Metagenomic sequence data are being used to assemble genomes of bacteria in natural communities. This has been successfully performed for the relatively restricted microbiota found in a subterranean acid mine drainage biofilm where near-complete bacterial genomes were reconstructed for two previously uncultured bacteria (Tyson et al. 2004). More ambitiously, Venter et al. (2004) randomly sequenced the metagenome of water specimens collected from the Sargasso Sea. Over one trillion base pairs of DNA were sequenced which were found to be derived from 1800 species including 148 not previously characterized. More than one million novel genes were found. However, it proved difficult to reconstruct complete genomes from these data because of the number of closely related species present and the large numbers of mobile elements with high levels of sequence similarity. A number of software tools are now available for genome assembly from metagenomic data, both as stand-alone programs or as online resources (Hunter et al. 2014) although it remains a challenging task.

Metagenome analyses allow an appreciation of the genetic potential of a microbial community but no indication of actual activity. Analysis of the mRNAs present in a sample shows which genes are currently being expressed and thus the functional activity of the

microbiota. RNA is extracted directly from the sample and then enriched for mRNA and reverse transcribed. The DNA is then fragmented and sequenced using next generation methods. After removal of contaminating and repetitive sequences, the reads are then mapped to reference sequences in nucleotide databases in order to identify genes and operons which have been expressed (Carvalhais et al. 2012). This approach has been successfully used with oral samples from subjects with periodontitis and caries (Duran-Pinedo et al. 2014; Simon-Soro et al. 2014).

1.6 Bacterial–bacterial communication

Originally thought of as simple dumb solitary creatures, it is now known that bacteria live together in communities with a number of features in common with multicellular organisms. The basis for the cooperation of individual bacterial cells within a community is communication. Communication is mediated by the production of signaling molecules often generically described as quorum-sensing molecules, after the first bacterial signaling system to be described.

In many circumstances, the total number of bacterial cells in a community is important to the overall health of the community. In the environment, the availability of nutrients and external stresses are factors that cause the community to behave in a particular way. This may increase or decrease its overall rate of growth, become more motile to move to a new habitat to obtain nutrients and, once there, switch to a biofilm mode of growth to colonize the new environment. For pathogenic bacteria of exogenous source, bacterial–bacterial communication is particularly important. The first bacteria to colonize the host will necessarily be present in small numbers and will want to multiply without alerting the host to their presence in order to avoid the host's immune system. As virulence factors such as protein toxins are typically highly antigenic, the pathogen will not produce them until the community is sufficiently numerous to resist the host's defenses. Once this “quorum” has been achieved, the members of the community will turn on the production of their virulence genes in order to damage the host and cause disease.

The molecular basis for quorum sensing was first elucidated for the bioluminescent marine bacterium *Vibrio fischeri*. This organism is commonly found

as a symbiont in the light-producing organs of luminescent fish or squid. The signaling mechanism is a two-component system; the *luxI* gene produces autoinducer 1 (AI-1), an acyl homoserine lactone. This is produced constitutively, but when sufficient numbers of *V. fischeri* are present, the high concentration of AI-1 enables binding to the receptor, the product of *luxR*, which activates transcription of the luciferase operon and leads to production of the light-emitting compounds.

Following its discovery in *V. fischeri*, AI-1 analogs have been found in a wide range of Gram-negative bacteria. The AI-1 of a particular species is normally specific to that species so that cross-talk is avoided within multispecies communities.

Gram-positive bacteria also produce signaling molecules, but those so far described have all been peptides derived from larger precursors by posttranslational modification. An important group of signaling molecules in Gram-positives is of those that induce competence. Competence is the ability of bacteria to take up DNA present in the environment. This is an important mechanism of genetic exchange and is particularly common among members of the oral microbiota such as the oral streptococci. In many streptococci, the precursor molecule is Com C which is modified as it is transported out of the cell by the ComAB transporter. The signaling molecule itself is the C-terminal end of Com C and is termed the competence-stimulating peptide (CSP). When the bacterial numbers reach their quorum, CSP binds to the receptor, the histidine kinase Com D, which then stimulates the response regulator Com E.

In addition to the species-specific quorum-sensing mechanisms described above, bacteria also make use of nonspecific systems that allow general communication between bacteria in communities, across species barriers. One such molecule, AI-2, is produced by, and can be detected by, a wide range of both Gram-negative and Gram-positive bacteria. AI-2 forms spontaneously from 4,5-dihydroxy-2,3-pentanedione (DPD), which is a product of the Lux S enzyme in the catabolism of S-ribosylhomocysteine. A number of oral bacteria have been shown to produce AI-2 and it has been found to have an important role in dental plaque formation. For example, *Streptococcus oralis* and *Actinomyces naeslundii* are known to coaggregate early in the development of dental plaque biofilms and grow together in *in vitro* models, forming a profuse plaque with physical

interaction between cells of the two species. A *luxS* mutant of *S. oralis* that did not produce AI-2, however, did not form such biofilms with *A. naeslundii*, while the mutualistic activity was restored by *luxS* complementation (Rickard et al. 2006). Quorum sensing is thus a central mechanism in bacterial metabolism, with particular importance in biofilm formation. The use of quorum-sensing inhibitors has potential for use as antibiofilm agents (Brackman and Coenye 2015).

Another group of bacterial cell-signaling molecules are the family proteins related to resuscitation-promoting factors (Rpf). Originally discovered in *Micrococcus luteus* where they were able to revive *M. luteus* cells that had entered a dormant phase, they were subsequently found to be widespread among members of the phylum *Actinobacteria*, the High G+C Gram-positives (Mukamolova et al. 1998). Interestingly, the growth of *Mycobacterium tuberculosis*, which is normally extremely slow *in vitro*, is greatly stimulated by Rpf. Rpf is a protein, structurally similar to lysozyme, which can exert its effects at extremely low concentrations. It has therefore been termed a bacterial cytokine because of its resemblance to mammalian cytokines that have similar properties. The molecular basis of its action has yet to be determined but it would appear to cleave the peptidoglycan of dormant cells and either release a second messenger or physically allow the cells to resume growth. Peptidoglycan fragments, muropeptides, have recently been recognized to be important mediators of communication both between bacteria and between bacteria and eukaryotes (Dworkin 2014).

Novel mechanisms of bacterial communication are being discovered all the time, and it is extremely likely that a network of sophisticated interactions exists among the bacterial community in dental plaque. Many of these are clearly relevant to endodontic infection and the survival of bacteria under restorations and in the treated root canal. The realization that vegetative bacterial cells can go into a dormant state, distinct from endospore production, and survive for many years may explain how bacteria survive under restorations or despite calcium hydroxide treatment in the root canal. Furthermore, a change in the environment may stimulate the production of broad-range growth stimulation factors that cause the community to undergo rapid growth, causing damage to the affected tooth and pain to the patient.

1.7 Host–bacterial interactions

All plants and animals are colonized by bacteria. Mammals are born sterile but extremely quickly become colonized with the microbiota characteristic for their species. The commensal microbiota associated with mammals has evolved over millions of years, and it is possible to reconstruct the evolution of the commensal microbiota in parallel with each mammalian host, the phenomenon of cospeciation. Thus, for the majority of bacteria found in the human mouth, there are versions of that organism found in other animals. For example, among the mutans group streptococci, associated with dental caries, *S. mutans* and *S. sobrinus* are found in humans, *S. ferus* and *S. rattus* in rats, *S. cricetus* in hamsters, and so on.

The recognition that human cells make up only around one-third of all cells in the body, with the majority of the remainder Bacteria (American Academy of Microbiology 2014), led to initiatives to describe the microbial populations and their genomes at various body sites, principally the National Institutes of Health-funded Human Microbiome Project (HMP) (Human Microbiome Project Consortium 2012a). The principal findings of the HMP to date have been that each body site has its own characteristic microbiota and that individuals have their own microbiome, which is relatively stable over time (Human Microbiome Project Consortium 2012b; Ding and Schloss 2014).

Our normal microbiota protects us from exogenous infection via the phenomenon of colonization resistance. All external surfaces of the body are normally covered in bacteria and thus potential binding sites for exogenous pathogens are blocked. In addition, members of the normal microbiota can produce antimicrobial substances that inhibit the growth of other organisms. However, if the commensal microbiota is disturbed then infection can result. For example, it is well known that treatment with antibiotics can disrupt the normal microbiota to such a degree that opportunistic infection with other organisms such as coliform bacteria or the yeast *Candida albicans* can occur. Vaginal thrush and antibiotic sore tongue are examples of such conditions.

The presence of the normal microbiota is essential for the proper development of the gut. The intestinal microbiota is highly diverse, with over 1000 bacterial species present. A commonly found species, *Bacteroides thetaiotaomicron*, has profound effects on

the development of the blood supply to the gut. In germ-free mice, introduction of *B. thetaiotaomicron* induced intestinal angiogenesis (Stappenbeck et al. 2002). Interestingly, the genome of *B. thetaiotaomicron* includes an unusually high number of genes encoding signaling molecules of both the one- and two-component types (Xu et al. 2003). The mucin-degrading bacterial species *Akkermansia muciniphila* is associated with gut health and its numbers are depleted in inflammatory bowel disease (Belzer and de Vos 2012). It is one of a number of species that have been found to be health associated and regarded as beneficial.

1.8 Complex infectious diseases

“Classic” infectious diseases normally occur when a pathogen infects a susceptible host and produces a specific virulence factor that damages the host in a characteristic way, causing the signs and symptoms of the disease. For many diseases, particularly those associated with the mucous membranes, no single pathogen has been identified, but instead the disease appears to be the result of an aberrant interaction between the host and its normal resident microbiota. These so-called complex infectious diseases include the inflammatory bowel diseases and oral infections such as chronic periodontitis and, to some extent, endodontic infections. It has been suggested that a change in composition of the microbiome to one that is in a state of dysbiosis may have a role in obesity, diabetes, mental health, and other conditions (Devaraj et al. 2013; Clarke et al. 2014). The question that remains to be answered is whether a dysbiotic microbiome is a primary driver of disease or whether it is a result of the disease process.

Host susceptibility is of primary importance in these diseases, but typically the susceptibility is conferred by multiple genes with no single genotype responsible. For oral diseases such as periodontitis, the genes responsible have yet to be discovered although there is growing evidence that increased susceptibility is caused by subtle differences in the immune and inflammatory responses. For example, genetic polymorphisms associated with cytokines such as interleukin-1 have been identified, which are associated with increased cytokine secretion and severity of chronic inflammatory disease (Brett et al. 2005). Another key factor is the environment in its widest sense. Host

factors such as stress are known to contribute to the severity of complex diseases, presumably by adversely affecting the immune system. Diet and social factors such as smoking can also be important, particularly in the principal oral diseases such as dental caries and the periodontal diseases.

It is likely that in the investigation of oral infections and diseases, we have clung too long to the classic infectious disease model and have sought single infectious causes for them in the hope that antimicrobials could be used in a targeted way to treat them. It must be remembered, however, that these diseases are bacterial diseases and the presence of the normal microbiota is required. By mechanisms as yet unknown, it appears that the communication and cooperation between the host and its commensal microbiota breaks down, resulting in damage to the host. Much work on these diseases is therefore currently being focused on better understanding health, the question being that if the human gut is colonized by so many bacteria with the potential to cause disease, how do the majority of individuals remain healthy? Better understanding of how this healthy balance is maintained will permit insights into how disease arises when the homeostasis breaks down. It may also be possible to influence the host–microbiome interaction with probiotic bacteria or by the administration of prebiotics to increase the relative proportions of beneficial bacteria (Claes et al. 2014). A more extreme treatment for patients with severe dysbiosis such as that seen in pseudomembranous colitis due to *Clostridium difficile* is a fecal transplant from a healthy donor, which has been shown to result in an excellent clinical response (Rao and Young 2015).

1.9 The future

We are still in the early years of the twenty-first century, so what do we have to look forward to in terms of how microbiology will impact on our understanding of infectious disease, including oral and endodontic infections? The Human Microbiome Project is providing an enormous bank of data on the composition of the human-associated microbiota and its genetic potential. The next challenge is to exploit these data to devise novel preventive and therapeutic strategies. It may be possible, for example, to construct a mixture of beneficial bacteria as an alternative to fecal

transplants, to be used to prevent, or reverse, dysbiosis. From the host perspective, advances in genetics will undoubtedly give us a far better understanding of individuals' susceptibility to disease and the challenge will be put this into context with new knowledge of the genetic potential of the commensal microbiota in order to predict and influence interactions among the host, its microbiome, and the environment.

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Chapter 2

Diagnosis, Epidemiology, and Global Impact of Endodontic Infections

Dag Ørstavik

- 2.1 **Endodontic disease: irritation, inflammation, and infection of the pulp and periapical tissues**
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2.1 Endodontic disease: irritation, inflammation, and infection of the pulp and periapical tissues

Endodontics deals with diseases of the pulp–dentin organ and the periapical tissues. For practical purposes, these are infectious processes. Noninfectious conditions affecting the pulp or apical periodontium are much rarer and are seldom dealt with by specific endodontic treatment; however, they represent important differential diagnostic challenges.

The sources of pulpal and apical periodontal infections are numerous. Traditionally, endodontic disease has been seen as a sequel to dental caries; however,

bacteria find their way to a vulnerable pulp in many other instances as well. Dental trauma is one well-known situation, so is pulp damage and infection following preparation and restoration of teeth. Low-grade irritation of pulpal nervous elements can occur following attrition and erosion, sometimes developing into pulpal necrosis and infection.

Historically, the focus has been on the inflammatory reactions of the pulp and periapical tissues, associating clinical disease with the tissue response. The inflammatory reactions have been related to infection, but at times they have been related to tissue damage during treatment and to the toxic effects of medications and materials. It is clearly an improvement in the

concept of diagnosis and treatment planning that there has been a shift towards stressing the level and extent of the infectious process, rather than wild-guessing the type and degree of the inflammatory reaction. Inflammation of the pulp and periapical tissues is a sign of infection; clinically progressing disease is hardly ever caused by trauma or materials. This concept has been productive because virtually all successful therapeutic measures are directed towards combating or preventing infection, with reduced or eliminated inflammation following as a consequence. Moreover, the concept of endodontic diseases as infections has implications for public oral health assessment in general, and places the association of local infectious disease in perspective relative to local and regional (Ricucci and Bergenholtz 2003) systemic health issues, particularly cardiovascular disease (Caplan et al. 2006; Joshipura et al. 2006; Cotti and Mercurio 2015). In this context, it is important to relate epidemiologic aspects of pulpal and periapical disease to endodontic microbiology.

2.2 Primary diagnostic criteria: subjective symptoms and radiographic changes

2.2.1 Pulpal involvement

Initial pulpal infection is recognized primarily by clinical symptoms or through explorative excavation of involved dentin. While conventional radiography may suggest that a resorptive or carious process is impinging on the pulp, such methods do not allow definitive assessment of pulpal involvement.

Traditionally, it has been held that there is only a weak association between clinical features and the histologic characteristics of pulpitis (Cisneros-Cabello and Segura-Egea 2005; Giuroiu et al. 2015). However, in a recent study, Ricucci et al. (2014) were quite successful in correlating pulpal history and symptomatology with histology and histobacteriology in 95 human teeth. The diagnosis of pulpal infection/inflammation is therefore largely an operational one: based on experience, and on knowledge of the underlying biologic processes, pulpitis is categorized as either reversible or irreversible. This scheme sidesteps the need to give a precise description of the extent and severity of inflammation in the pulp, but it allows for treatment decisions based on the extent of microbial contamination or infection. It is assumed that in the case

of reversible pulpitis, pulp vitality may be preserved with proper treatment; irreversible pulpitis implies that no treatment short of pulp extirpation and root filling can eliminate the disease. Briefly, reversible pulpitis causes clinical symptoms of short duration (seconds) and only when irritated by external stimuli, and the pulp proper is either not exposed or traumatically exposed for a short period only (< 2 days) (Heide and Kerekes 1987). By contrast, irreversible pulpitis gives rise to symptoms of longer duration (minutes) that may also occur spontaneously, and an exposure of the pulp to the oral environment through caries, fractures, or cracks is suspected or confirmed. This concept is supported by clinical experience and experiments (Rodd and Boissonade 2000; Sigurdsson 2003; Iqbal et al. 2007; Ricucci et al. 2014) and by experimental studies on the effects of pulpal inflammation on nerve activity (Rodd and Boissonade 2000; Bletsas et al. 2006; Kokkas et al. 2007). Box 2.1 lists the salient clinical signs of irreversible pulpitis (i.e., infection of the pulp necessitating endodontic treatment by root filling).

Box 2.1 Clinical characteristics of irreversible pulpitis

- Severe pain necessitating dental emergency treatment
- A history of repeated pain episodes
- Self-medication with analgesics
- Pain lingering after end of stimulus
- Sleep or work affected
- Supporting findings: positive, sometimes exaggerated pain on thermal or electrical stimulation; tooth localization difficult; percussion test largely negative

Sometimes, a carious process may have reached the pulp without any symptoms. Traditionally, and in most settings in a dental office, this is considered an irreversible pulpitis (i.e., the tooth will need endodontic treatment; Bjørndal et al. 2010). However, modern materials and aseptic techniques may also provide predictable results from pulp-preserving approaches in these clinical situations (Bogen et al. 2008; Marques et al. 2015), but such approaches can be highly operator-sensitive (Miles et al. 2010) and therefore may not yet be recommended as standard practice.

Sensitivity testing by temperature or electrical pulses can give reasonably accurate assessment of nerve tissue activity in the pulp, but relating such recordings to the degree of pulpal inflammation is difficult considering the large variation in such measurements and their dependence on other clinical parameters associated with the tooth (Fischer et al. 1991; Chen and Abbott 2009; Alomari et al. 2011; Mejare et al. 2012).

Radiography, including cone-beam radiographic techniques, is useful in special circumstances, such as for detection of internal and external cervical resorption (Celikten et al. 2014; Kalender et al. 2014; Ven-skutonis et al. 2014; Creanga et al. 2015; Dogramaci et al. 2015; Mavridou et al. 2016), which often affects the dental pulp. Pulp calcifications (diffuse and globular) and obliteration as seen radiographically may give indications of the physiologic state of the pulp, but little information about pulpal infection or inflammation.

In summary, a clinical pulpal diagnosis is most often made based on anamnestic and subjective data supported by sensitivity testing and caries excavation.

2.2.2 Periapical diagnosis

When the infection of the dental pulp affects the periodontium, apical periodontitis occurs. Pathologically, the inflammation is organized as a granuloma that may or may not develop a radicular cyst as a sequela (Nair 2008). Periapical disease also has a significant clinical component. In comparison with symptomatic pulpitis, symptomatic apical periodontitis is typically characterized by dull rather than sharp pain, and positive percussion and palpation tests (Iqbal et al. 2007; Sigurdsson 2008). Total infection of the pulp with virulent organisms can give rise to acute apical abscess, a very painful and potentially harmful condition (Antunes et al. 2013; Chunduri et al. 2013; Moazzam et al. 2015), exemplifying a disease that historically defined the dental profession. Longstanding pulp infections with chronic apical lesions can exacerbate with the same symptomatic apical periodontitis or acute apical abscess. Apart from distinguishing such conditions from marginal periodontal inflammation, and in particular a periodontal abscess, they are seldom difficult to diagnose.

Asymptomatic apical periodontitis is, on the other hand, dependent on radiographic signs for diagnosis.

In its early stages and during healing, this may be very difficult, whereas a well-established, asymptomatic periapical lesion is a simple condition to identify on radiographs (Ørstavik and Pitt Ford 2008). In teleologic terms, an infected root canal of a tooth is probably perceived by the body as a risk zone for invasion by (life-threatening) microbes. A defense region is then established in which the tissue architecture is changed to prepare for the containment of invading microorganisms (Ørstavik and Larheim 2008). Bone is gradually replaced by granulomatous tissue with vascular and cellular components mobilized for host defense. These initial events produce changes in bone structure at the apex, which may be very hard to detect by periapical radiography (Brynolf 1967), and they may occur with teeth that still have vitality or at least neural activity in the pulp (Figure 2.1). When periapical tissue remodeling has reached a state of complete granulomatous transformation, the lesion is very characteristic and easily diagnosed on the radiograph, particularly when a cortical plate is affected (Figure 2.2). If such a tooth does not respond to sensitivity testing, a diagnosis of pulpal infection and apical periodontitis is certain, and treatment options instantly available. On the other hand, there may be total pulp necrosis and no infection or associated inflammation at the apex, such as when the pulp is devitalized by traumatic injury (Sundqvist 1976).

The chronic development of apical periodontitis may be totally without symptoms, in which case the term asymptomatic apical periodontitis is appropriate. However, symptoms may occur at any stage during the process, ranging from barely perceptible tenderness to the acute symptoms described earlier.

In summary, chronic asymptomatic apical periodontitis needs radiography for detection; symptomatic and acute phases are diagnosed by clinical symptoms and signs.

2.3 Pulpal inflammation and infection: public health consequences

The clinical aspects of endodontic diseases may be serious and with some consequences for individual and public health. Pulpitis and apical periodontitis are traditionally categorized under “caries and its sequelae,” and it is certainly true that deep carious lesions

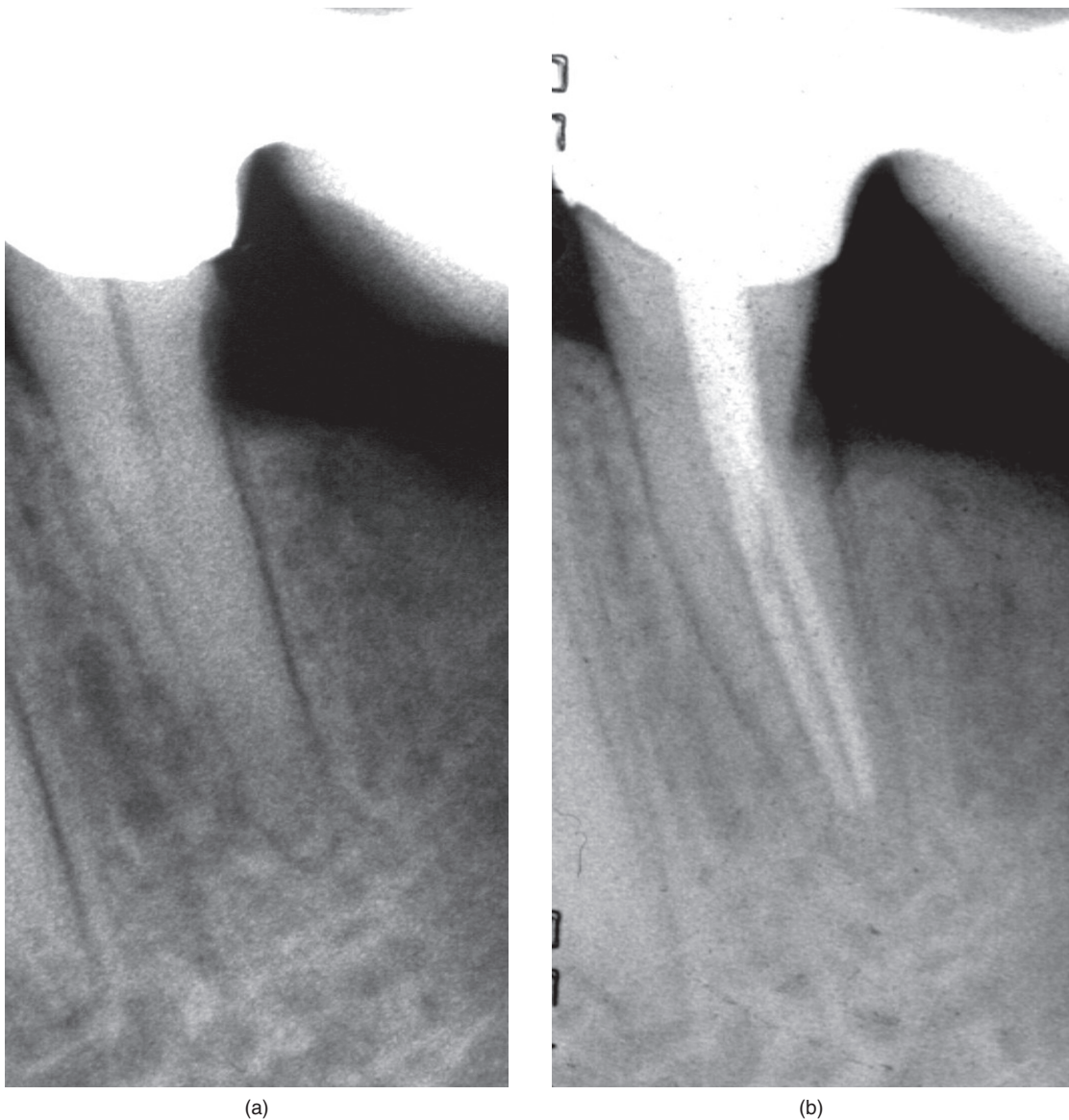


Fig. 2.1 Minimal bone structural changes at the apex in conjunction with chronic pulpitis (a), necessitating endodontic treatment (b).

are indicators of pulpal and periapical inflammation. This is a confounding factor for assessments of the relative importance of these conditions in the overall incidence and prevalence of orofacial pain. Pulpitis may be very painful and lead to loss of quality of life (Constante et al. 2012). It may also cause absence from work and loss of income (Miotto et al. 2012).

It is unfortunate that pulpal pain is pooled with other tooth-related pain and often with the whole specter of orofacial pain conditions in surveys and screening studies. If one assumes that emergency treatment of dental caries is initiated by pulpal pain and thus falls under the category of endodontic disease, symptomatic irreversible pulpitis and apical periodontitis

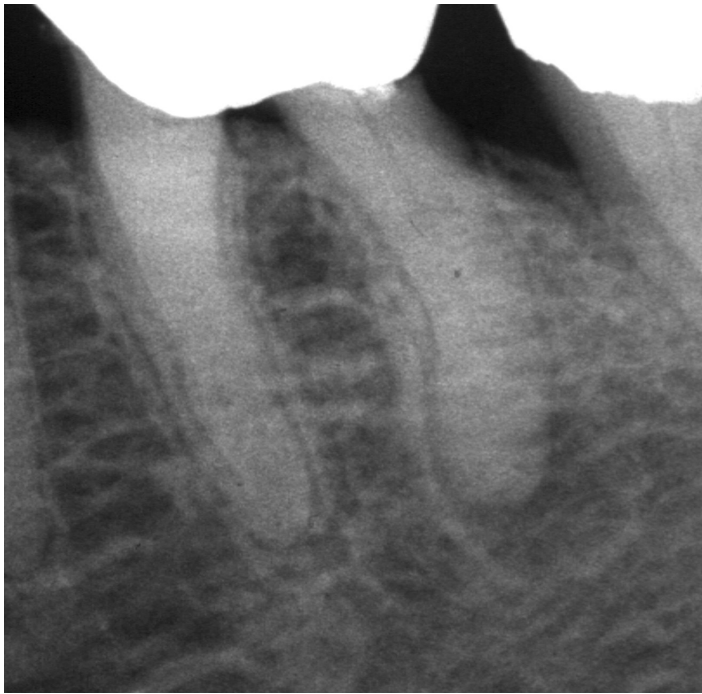


Fig. 2.2 Chronic apical periodontitis: incipient at mesial root, established at distal root of mandibular left first molar.

dominate as sources for acute dental pain in children and adults (Zeng et al. 1994; Lygidakis et al. 1998; Tulip and Palmer 2008). This may be debilitating to the patient and lead to absence from work and involvement of costly health services. While it is known that emergency dental services are in great demand in most countries, in urban as well as rural areas, there is scant information on the actual incidence and prevalence of acute pulpal and apical periodontal disease. Therefore, one can only speculate that there is still, even in communities with well-developed dental services, a significant impact on the general well-being by acute pulpal and periodontal conditions (Sindet-Pedersen et al. 1985; Richardson 2005; Cope et al. 2014).

It seems that psychologic factors influence the incidence and severity of orofacial pain including pulpal and periapical pain (Aggarwal et al. 2010). Therefore it is especially important for susceptible individuals to have conditions that cause acute dental pain treated quickly and efficiently.

A frequently overlooked situation is the association of pulpal and apical disease with tooth loss in the

elderly and in highly restored dentitions (Dikbas et al. 2013). Whereas marginal periodontal disease is generally accepted as a significant cause of tooth loss, pulpal and apical diseases are important causes for extraction (Eckerbom et al. 1992; Lee et al. 2015) and may dominate after the age of approximately 50 years (Eriksen 1991).

The tooth with pulpitis is obviously in danger of becoming infected and developing apical periodontitis. Correct and prompt treatment of the acute situation is therefore important, not only to curb the pain and to re-establish a functional tooth, but also to reduce or eliminate the risk for the insidious spreading of the infection and the emergence of a periapical lesion. It has been known for a very long time that the prognosis for treatment of apical periodontitis is much poorer than expected treatment outcome after vital pulpectomy (see Chapter 15). Early detection and root canal treatment of teeth at definitive risk of developing root canal infection are therefore essential. Failure to provide adequate treatment early will facilitate the development of an infection (Figure 2.3), which will reduce the prognosis.