Review Article

Changing concepts in caries microbiology

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ABSTRACT: Concepts and beliefs about the cause of dental caries have evolved over many centuries, with the involvement of microorganisms being recognized since the late 1800s. A main thrust of enquiry since then has been to tackle the question of the relative importance of different bacteria in the disease and this article will consider how technical advances in our ability to identify, cultivate and count different species has influenced our understanding. Over the last decade, molecular biological approaches have had a major impact on views of the relative contribution of particular species of plaque bacteria to the caries process. At a more detailed level, molecular genetic studies of species such as *Streptococcus mutans* have given new insights into the way in which particular genes and the functions that they encode may affect virulence. (*Am J Dent* 2009;22:304-310).

CLINICAL SIGNIFICANCE: An understanding of the bacteria involved in the initiation and progression of dental caries is essential for a rational approach to developing microbiological markers of risk, monitoring the effect of interventions and devising control measures.

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Introduction

The first technical advance influencing our understanding of oral microbiology came with the development of microscopes by Robert Hook and Antonie van Leeuwenhoek in the 17th century. van Leeuwenhoek recorded how he was fascinated by the seething activity he could observe when he examined scrapings from his teeth, and took great delight in the variety of shapes, sizes and movement of the 'little animalcules' that he saw, including what we now recognize as streptococci, fusobacteria and spirochetes. van Leeuwenhoek noted not just this diversity in his own mouth, but observed that everyone he examined harbored a different mixture of types of organism, thus setting the scene for subsequent discoveries in oral microbiology. Although van Leeuwenhoek appears not to have made any connection between what he observed and dental disease, 200 years later his observation of bacteria was central to the "germ theory" of disease that advanced with the development of techniques for growing pure cultures of bacteria in the laboratory. The latter part of the 19th century saw rapid advances in the quest to identify the causative organisms of infectious diseases, with the bacteria responsible for diseases including anthrax, plague, dysentery, cholera and tuberculosis isolated and cultured in the laboratories of Pasteur, Koch and others. This pioneering work and the formulation of "Koch's Postulates" that provided a framework for the implication of specific etiological agents was an essential precursor to studying the transmission and spread of disease, identifying sources of outbreaks, developing targeted vaccines and selecting appropriate chemotherapeutic agents.[1]

With these practical advances in bacterial characterization and identification of the etiology of many infections, it was natural that interest should be aroused in determining the specific etiology of other diseases, including dental caries. The International Medical Congress held in London in 1881 was clearly a major event, with leading figures including Pasteur, Koch and Lister attending. At that same congress two London dentists, Underwood and Milles, gave a paper describing the microscopic observation of "germs" in decaying teeth and experiments in which they incubated extracted teeth in test tubes and looked for damage to the enamel.[2] They found that enamel dissolution occurred only if there was both a source of carbohydrate (chewed bread in their experiments) and live germs, while killing of germs by a phenolic solution prevented any damage to the enamel. Nothing more is known of their investigations but their concept of the three-way interaction between bacteria, carbohydrate and teeth was developed and firmly established by Willoughby D. Miller, an American working in the same institute as Koch in Berlin.[3] Miller came from Ohio and started out as a physicist who became involved in dentistry after he moved to Berlin, though he returned to train in Philadelphia. He later rose to be the first non-German professor at the University of Berlin and treated the young Kaiser, but his lasting scientific achievements are encapsulated in his book *The Micro-organisms of the Human Mouth*.[4] While giving ample credit to earlier workers, Miller was the first to unify the ideas of the time by advancing his "chemicoparasitic theory" identifying the essential conjunction of bacteria and fermentable carbohydrate to generate the acid that resulted in the demineralization of enamel. While Miller must certainly have been immersed in the contemporary quest for etiological agents by colleagues such as Koch, he himself made little progress and acknowledged "… whether there is any one bacterium which may *always* be found in decayed dentin, and which might therefore be entitled to the name of *the* bacterium of tooth decay, or whether there are various kinds which occur with considerable constancy, we are not able to say." Miller's own observations were largely based on microscopy, which clearly does not have the discriminatory power to identify particular species and has an inherent bias towards recognition of distinctive shapes, therefore the emphasis in his writing is on the thread-like *Leptothrix*.

However, rapid progress was being made in techniques for growing bacteria in pure culture so that the properties of individual species could be characterized. By the turn of the century, Goadby[5] could argue that both acidogenic and proteolytic bacteria were involved in the caries process but stated confidently "… there is no specific organism of dental

decay". This of course did not stop others seeking particular causative agents, in the hope of bringing dental microbiology into line with what might be called the classical medical microbiology model based on a specific etiology for each disease. In that model, the causative organisms would be expected to be present at the point of pathological damage. However, as Konig[6] pointed out in an introductory essay when Miller's book was reprinted in its entirety, Miller did not believe that dental plaque was of any significance, taking the view that the important acid was produced by salivary bacteria. Some of his contemporaries, however, were convinced that local production of acid within deposits on teeth was essential for decay.[7] Here we see another example of how a practical approach can influence our depth of understanding: in this case, the choice of sampling site and the importance of identifying the bacteria at the actual point of attack. Of equal importance to sampling site is the time at which a sample is taken with respect to progression of the caries lesion and the recognition of this led to the next major breakthrough.

DISCOVERY OF *STREPTOCOCCUS MUTANS*

Most early workers examined advanced caries lesions but a different approach was taken by the medical microbiologist Clarke.[8] In the 1920s, the most popular villain was *Bacillus acidophilus odontolyticus* (which we would now know as a *Lactobacillus*) but Clarke showed that this was found only in established lesions where its growth is favored by the low pH. Clarke also exposed a bias in the commonly-used culture techniques which used media at a low pH. These are biased towards the selective growth of *Lactobacillus* spp. but when he plated his samples at pH 7, he found that early lesions were dominated by an organism to which he gave the name *Streptococcus mutans*. Clarke's contribution was thus several-fold. In addition to describing *S. mutans*, he introduced the concept of microbial succession with different bacteria being dominant at different stages of the caries process and raised the question of selective bias in detecting bacteria present.[9] Although not explicitly stated, his insight that different bacteria make different contributions at different stages of disease, with early participants setting up conditions that favor later invaders, is an important factor in our current understanding. Clarke quit dental research a year later[9] and his work went largely unregarded until the late 1950s when new approaches to animal models of caries, based on the selective use of antibiotics to modify the oral microflora and the introduction of isolation chambers where germ-free animals could be raised, refocused attention on the streptococci.

ANIMAL EXPERIMENTS

Progress in this era has been thoroughly reviewed.[10] Experiments in animals revealed a hierarchy of cariogenicity, in which various species of streptococci could be ranked according to their ability to induce caries in rats or hamsters fed a sugar-rich diet. Top of the list were the mutans streptococci, though it was some years before it was appreciated that only *S. mutans* and *Streptococcus sobrinus* are regularly found in humans, while *Streptococcus ratti* and *Streptococcus criceti* are characteristically isolated from rats and hamsters respectively.

Since rats do not naturally carry significant numbers of *S. mutans* or *S. sobrinus*, an animal model of caries could be developed by infecting rats with strains isolated from humans, and one known as *S. mutans* 6715 (the fifteenth isolate from an experiment at NIH in 1967) that was later reclassified as *S. sobrinus* became the focus of attention. In many ways, this strain was more attractive for experimental work than *S. mutans*, being more acidogenic and more adhesive in the presence of sucrose, though it does have the disadvantage of not being easy to manipulate genetically. The model system based upon *S. sobrinus* infection of rats made it possible to explore the importance of various possible virulence factors and to test the feasibility of immunizing against caries, using vaccines based on the glucosyltransferases (GTF) that synthesize sticky glucans from sucrose. However, while such model systems are of enormous value in taking research forward, there is a need for continual vigilance to ensure that the various components of the system (bacterium, animal, diet) truly represent the features of the human disease in which we are interested. Taking the first of these, the realization that *S. sobrinus* occurs much less frequently than *S. mutans* in humans meant that the latter was the more obvious target for developing a vaccine for use in humans. Rats' teeth also differ in morphology from those of humans, and suffer from extensive sucrose-dependent decay of smooth surfaces. This emphasis on sucrose-dependent adhesion led to a great amount of attention being focused on the GTF.[11,12] The glucan synthesized by glucans can have a powerful influence on the properties of plaque but, while being fascinating enzymes in their own right, it is not at all certain that GTF are anything like as significant in human caries as they are in the rat model.[13,14] For a start, smooth surface caries is much rarer in humans and the essential involvement of sucrose is far from clear.[15] Taken together, these observations may explain the failure of a GTF vaccine in macaque monkeys, which have tooth morphology and oral microflora close to that of humans.[16] There is, however, some evidence that the GTF of strains of *S. mutans* from caries-active individuals synthesize more water insoluble glucan than strains from caries-free subjects and this will be interesting to explore in the light of recent discoveries about variation within the species.[17,18]

MICROBIOLOGY OF HUMAN CARIES – MUTANS STREPTOCOCCI

Animal experiments played a crucial part in focusing attention on the mutans streptococci as cariogenic organisms, and this view was reinforced by the fact that they possess properties believed to contribute to virulence, notably acid production and sucrose-dependent adherence to hard surfaces.[19] There was thus a widely-accepted shift in opinion from the Non-Specific Plaque Hypothesis (which regards all plaque bacteria as contributing to disease) to the Specific Plaque Hypothesis, which considers a restricted subset of species to be responsible for disease.[20] Testing the latter demanded a simple and efficient means of identifying and counting the species of interest, so considerable effort went into developing a selective growth medium for *S. mutans*. The quest continues to this day because of the difficulty of finding a perfect selective medium that will suppress growth of all bacteria other than the one sought, at the same time allowing all strains or variants of the targeted spe-

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cies to be isolated. The two most successful and widely adopted selective media have been MSB and TYCSB.[21,22] A large number of cross-sectional and longitudinal studies, mostly using these media, have confirmed a strong association between mutans streptococci and caries and a systematic review found 2,730 papers on the topic.[23] It has long been recognized that neither of these media is perfect and also that it is not possible to reliably distinguish *S. mutans* from *S. sobrinus* on colony morphology alone so their relative importance could not be distinguished.[24] In those cases where identification to species level using cultural methods has been performed, *S. mutans* is by far the more common and is carried by over 98% of adults so its association with caries is clear, though *S. sobrinus* has been reported to be found in only 5-40% of individuals in different countries.[25] A number of studies have found that children with high caries activity are more likely to carry both *S. mutans* and *S. sobrinus*, linking the latter to extensive caries and/or smooth surface lesions.[23,26-30]

How reliable are these culture-based findings? Certainly, numerous independent studies have allowed consensus that mutans streptococci are associated with caries.[23] Although the great majority of these studies could not have distinguished the two species, 20 years ago Loesche[25] concluded on the evidence available that *S. mutans* was by far the more important. However, non-cultural methods for detecting *S. sobrinus* based on use of monoclonal antibodies, "checkerboard" DNA hybridization or PCR all reveal the presence of a much greater frequency of *S. sobrinus* detection than did cultural methods indicating that it is seriously under-represented on the selective media.[31-34] So what else can non-cultural methods tell us about results obtained with selective media? One recent study using 16S rDNA cloning and sequencing techniques for identification found that of 21 colonies with the morphology typical of mutans streptococci on the commonly used MSB agar were not, in fact, mutans streptococci but identified as *S. anginosus, S. sanguinis* and *Pantoea agglomerans*.[35] Invaluable though the selective media approaches were, it is clear that they provided an incomplete picture because of their inability to distinguish species. More important, perhaps, is the risk of making a Type II error. In other words, this is the error of failing to observe a difference when in truth there is one. By focusing on *S. mutans*, the possibility that other bacteria might also show an equally strong association with caries was largely set aside.

NON-MUTANS STREPTOCOCCI

In his pioneering work, Clarke observed that caries sometimes develops in the absence of any detectable *S. mutans*. This finding suggests that other species, or combinations of species, could produce similar amounts of acid to *S. mutans* and hence cause disease. By using non-selective media to characterize the predominant cultivable flora in health and disease, van Houte *et al*[36-40] concluded that the major organisms involved were the "low-pH non-mutans streptococci" which have subsequently been identified as atypical strains of common plaque species such as *S. mitis, S. sanguinis and S. intermedius*.

LACTOBACILLUS SPP. IN DENTAL CARIES

Lactobacilli can consistently be isolated from established caries lesions in humans but do not show an association with initiation of the disease.[23] There have been numerous attempts over the years to identify which species of lactobacilli are found in human caries but our understanding of the taxonomy of lactobacilli has been dramatically altered by the introduction of molecular biology techniques, particularly the construction of phylogenetic trees based on differences in sequence of 16s ribosomal RNA genes. It is now clear that a wide range of *Lactobacillus* species can be isolated from carious lesions and that these species are all found elsewhere in the body and/or in fermented foods.[41-45] The particular species found within the lesions will be largely dependent upon the environmental exposure (mainly diet) of the individual with a two-way exchange between oral and gut populations.[45] Because lactobacilli are so prevalent in fermented foods, we are constantly exposed to infection by species including ones present in probiotic foods such as yogurt marketed as beneficial for intestinal health.[46,47] There is no evidence for a uniquely oral species of *Lactobacillus, i.e.* no member of the genus has evolved to exploit the oral cavity as a habitat, in the way that some streptococci have done. Lactobacilli are thus not significant members of the normal plaque microflora in the absence of caries but are opportunistic invaders that take advantage of the low-pH conditions established by other bacteria in the depth of caries lesions.[48] Whether the same applies to the Bifidobacteria described below is as yet unclear.

BIFIDOBACTERIA

These anaerobic bacteria are taxonomically distant from streptococci but have very similar sugar metabolism and can produce lactic acid.[49] Although well-known as gut inhabitants, it is only quite recently that their occurrence in the mouth and possible association with caries has been recognized. This change in viewpoint is a consequence both of the introduction of molecular detection methods and the development of a selective medium using mupiromycin to suppress growth of other bacteria.[50] Several recent studies[51-55] have found significant numbers of *Bifidobacterium* in caries lesions and shown that their numbers correlate with those of other caries-associated bacteria though as yet virtually nothing is known about their origin or how they may contribute to the disease process. It is interesting to note that lactobacilli and bifidobacteria are the two major groups of bacteria found in probiotic products such as yogurts and other "functional foods". A number of reports have suggested that these may have potential as oral health care products on the basis of the fact that they interfere with the growth of plaque bacteria but it is important to realize that their potential to colonize may be quite different in healthy individuals and in those with active caries lesions.[56] The latter may provide more attractive ecological niches for the probiotic strains, but the introduction of new acidogenic species may not be a desirable outcome.

THE IMPACT OF MOLECULAR BIOLOGY

One of the first applications of nucleic-acid based technology that had a major impact in caries microbiology was the use of DNA-DNA hybridization to clarify the separation of the mutans streptococci into distinct species.[57] Further advances in taxonomy of streptococci and other genera have come from comparison of the sequences of 16S RNA genes though most

recently it has been found that carefully selected genes encoding conserved proteins provide improved resolution.[58-60] Two different molecular biology approaches have made a striking contribution to our understanding of the complexity of the microflora of dental caries, providing advantages over cultural approaches in the speed and precision of identification of the species present. Both are based upon analysis of DNA extracted from plaque taken at the sampling site. The results are therefore not affected by the ease with which particular species can be grown in the laboratory or by the selectivity of the growth media though interpretation of quantitative data can be complicated by concerns about the relative ease with which DNA is extracted from different species.

The first approach is based on DNA-DNA hybridization, in which DNA from the sample is tested for the presence of sequences that will bind to a defined panel of target DNA, usually immobilized on a membrane or "chip". The checkerboard hybridization technique developed at the Forsyth Institute in Boston allows detection and rough quantitation of a panel of species in a single experiment. In a pioneering report[52] examining 23 species, the strong association with caries for *S. mutans* was observed; *S. sobrinus* was not implicated though several other species were, including *Actinomyces gerencseriae* and a *Bifidobacterium*. A later study[61] with 82 species in the hybridization again found *S. mutans* and lactobacilli predominant, along with an *Actinomyces* and *Atopobium* spp. In contrast, *Abiotrophia defectiva*, *S. parasanguinis*, *S. mitis*, *S. oralis* and *S. sanguinis* exhibited an inverse relationship and can be regarded as beneficial species. A recent study,[62] detecting 300 different species, revealed further complexity and introduced new caries-associated species such as *Pseudoramibacter alactolyticus*. The Human Microbial Identification Microarray aims to eventually include in the hybridization panel some 600 oral bacterial species, of which over half have not yet been cultivated (http://mim.forsyth.org/index.html). Assuming that the panel really is fully comprehensive, this will be a valuable tool in examining complex microbial populations.

The second main approach that has been used to study the plaque microflora relies upon copying all the 16S ribosomal RNA genes present in a sample by polymerase chain reaction (PCR) and then determining the sequences of the amplified genes to determine what species were present in the sample. The PCR primers for amplification are designed to recognize conserved sequences flanking the 16S genes so that even novel or uncultured species can be detected. Several laboratories[42,43,63,64] have applied this approach to caries microbiology and each has demonstrated the complexity of the microflora and the broad range of species that may be considered caries-associated. Notable by their absence for these studies are *Bifidobacterium* spp. This can be explained by the fact that the 27f primer pair commonly used for 16SRNA amplification has mismatches with the *Bifidobacterium* sequence at 3/20 base pairs.[65,66] This would result in inefficient or complete failure to detect *Bifidobacterium* spp. in a plaque sample and illustrates the great difficulty of devising a technique that will provide a genuinely comprehensive overview of the population.

CARIES-ASSOCIATED BACTERIA

From the discussion above, it is apparent that each generation of identification techniques based on a different technology (microscopy, culture, selective media, molecular biology) has its limitations but has contributed to a steady accumulation of knowledge about the microbiology of the carious lesion. We are still only beginning to gain an appreciation of the extent of individual variation and the changes in plaque over time but we now have some very powerful tools at our disposal.[64,67,68] Clarke[8] first noted that caries could develop in the absence of detectable *S. mutans* but could say little about what other cariogenic bacteria might be present. The latest available report of array data found that 10% of subjects with rampant caries had no *S. mutans* and implicated a range of other associated species. Only some of these associated species are acidogenic and therefore contribute to damage. Others, such as the *Veillonella* spp. are not damaging but seem to be along for the ride and flourish on the high levels of lactate and succinate generated in the lesion.[69] We are rapidly accumulating an improved knowledge of the bacterial species that are caries-associated (which is not the same thing as cariogenic since are not all are acidogenic) and ones that are health-associated. Among the latter are species known to be capable of alkali generation that can have a strong influence on the final pH achieved in plaque.[70,71]

It is not the purpose of this article to consider the dynamics of bacterial succession and population changes in dental plaque as it shifts from being a harmless biofilm to being pathogenic since this aspect has been thoroughly reviewed recently.[40,72,73] Technical advances in continuous bacterial culture and the biofilm concept have been crucial to development of what has come to be known as "the ecological plaque hypothesis" in which repeated cycles of stress in the form of lowered pH due to consumption of fermentable carbohydrates lead to enrichment of acidogenic and aciduric species in plaque.[74] It is probably non-mutans streptococci and *Actinomyces* that contribute to the earliest stages while the mutans streptococci, bifidobacteria, lactobacilli and maybe others move in later to exploit the modified environment. At different stages in the caries process, and in different individuals, different combinations of bacteria may interact to produce the overall effect of a plaque with lowered pH.

IMPLICATIONS

Returning to the principles of disease causation introduced by Koch and others, we now have an extensive, but still very incomplete, picture of the microbial etiology of dental caries. We can answer Miller's query "whether there is any one bacterium which may always be found in decayed dentin, and which might therefore be entitled to the name of the bacterium of tooth decay" with a resounding NO. It is no longer appropriate to begin a paper (as so many do) with a statement that *S. mutans* is the principle etiological agent of tooth decay, when we know the inadequacies of such a simplistic statement. Does this mean that studies of *S. mutans* are no longer relevant? Far from it, but rather than focusing on its potential to act as a pathogen in isolation we need to appreciate its value as a marker organism for monitoring transmission, disease progression, risk and the success of efforts to reduce the cariogenic population.[75] In all these cases, we must remember that other bacteria may be behaving in exactly the same way as *S. mutans*, even if historically we have not measured them. Besides its value as an indicator of events in plaque, *S. mutans* is also of enormous value as a model organism and molecular genetics approaches have yielded considerable insights into its functions.[76]

MOLECULAR KOCH'S POSTULATES

The same type of logic that has been applied to the search for specific causative organisms in disease can be applied to the search for particular virulence factors that make an organism pathogenic.[77,78] Thus, it is possible to seek correlations between presence of a particular gene in an infecting organism and its virulence, or apply targeted gene knockout to examine the consequences.[76,79] Examples of the former approach is work on the relationships between the collagen-binding adhesin that is only found in some strains of *S. mutans* and research into different forms of GTF and childhood caries.[17,80] Gene knockout strategies, on the other hand, can only be exploited for *in vitro* tests or experimental animal systems. Knockouts have neatly shown the contribution of different GTF to adherence both *in vitro* and in rats.[81-83] However, as with the interpretation of experiments on the cariogenicity of bacterial species in caries, considerable caution is needed in extrapolating from rats to humans, particularly as it is known that results in rats are highly dependent on diet and the introduction of mutated strains can have unexpected effects on the balance of micro-organisms in plaque.[84,85] Banas *et al*[86] found that infecting rats with a mutant in which the *gbpA* gene for a glucan binding protein was inactivated induced the accumulation of mutants in which GTF genes were altered due to a chromosomal deletion. In other words, we now have evidence that not only do the relative proportions of different bacterial species fluctuate within plaque but even with a single species such as *S. mutans* there is genetic variation. This variation can be induced under experimental conditions but has been happening throughout the evolution of *S. mutans*, most probably in synchrony with human evolution, as has been found for other human pathogens.[87-89] The availability of the complete genome sequence of *S. mutans* strain UA159 has provided a starting point for comparative genomics and it seems that both chromosomal deletions and insertions due to horizontal gene transfer from other species are common events.[90-93] The extensive shuffling of core and dispensable genes means that no two isolates of *S. mutans* are likely to be the same so each of us is likely to carry several different genotypes, each with a distinctive set of genes which may modify its potential to interact with other plaque bacteria and contribute to caries. This means that we need to be cautious about assuming that what is true for UA159 is true for all strains of *S. mutans*; genes concluded to be associated with virulence in UA159 may be absent from other strains, which in turn will possess features missing from UA159. Furthermore, just as a deeper understanding of the complexity of plaque microflora makes us re-examine the question of targeting a single species (for example, in a caries vaccine) when we know that other combinations of species can produce the same pathogenic effect, so we must be careful in assuming that interference with a single gene's function will abolish virulence when some strains may achieve the same level of danger by deploying a different combination of properties.

Conclusion

The history of the study of caries over more than a century has been characterized by shifts in views of its microbial etiology. A major driver of these conceptual developments has been the introduction of new technical approaches, each of which has added to our overall knowledge but each of which has been found to have its limitations. Progress is thus a reiterative process in which we must continually be questioning and reinterpreting our results and formulating new hypotheses. In an earlier review Tanzer[10,94] drew our attention to an essay on "strong inference" that points out we should not just ask whether we should conclude a particular experiment proves something, but also what alternative hypotheses it is disproving. By applying this rigor and continual questioning to our studies of the complex and fascinating topic of the microbiology of caries we may eventually approach the truth, for the benefit of scientific progress and of our patients.

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