FACULTY OF DENTISTRY
Department of Odontology
Oral Microbiology

CENTRE
FOR
INTERNATIONAL HEALTH

IN VITRO
ANTIBACTERIAL EFFECT
OF THE CARISOLV®-2 SYSTEM

ROXANA IOANA DRAGHINCESCU D.D.S.

UNIVERSITY OF BERGEN – NORWAY
2004

"It is vital, as practicing dentists, to keep questioning what we do and why we do it. This is what ultimately sets the good clinicians apart from the rest."
(Banerjee, Watson and Kidd, 2000)

To my parents, Corina and Dan

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Thesis submitted to the Faculty of Dentistry, University of Bergen in partial fulfillment of the requirements for the degree of Master of Philosophy in Dentistry

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BERGEN, Norway
November 26, 2004   *Roxana Ioana Draghincescu*

SUMMARY

Carisolv® (MediTeam, Gøteborg, Sweden) is a product marketed to dissolve carious dentin thus enabling minimal invasive treatment of dental caries. Studies have reported antibacterial effect of the first generation of Carisolv. Recently the second generation (Carisolv-2) was introduced. No information has been published about the antimicrobial effect of Carisolv-2. **Aims**: **1.** To assess *in vitro* the *in situ* antimicrobial effect of the Carisolv-2 system on the bacteria in carious lesions. **2.** To compare the type and quantity of bacteria from non-carious dentin treated by conventional drilling or by the Carisolv-2 system. **3**. To evaluate the *in vitro* antimicrobial effect of the Carisolv-2 gel on selected cariogenic bacteria.

Materials and **Methods**: Twenty-four extracted permanent teeth with caries (class I and II) not involving the pulp were collected after the patients' consent and used in the study. Immediately after extraction and before Carisolv-2 application, one carious dentin sample was obtained from each of the lesions (samples 1) using slowly rotating sterile round burs. The teeth were then split aseptically in two halves along their long axis and through the middle of the carious lesions. The caries lesions of the two halves (n=48) were treated either with the Carisolv-2 system or by conventional drilling using sterile burs. Dental probes and a KaVo DIAGNOdent® laser apparatus were used to assess when caries-free dentin had been reached. Then, non-carious dentin was obtained in a standardized manner by sterile round burs from the bottom of the cavities after all carious dentin had been removed by drilling (samples 2) or Carisolv-2 treatment (samples 3). The dentin samples were weighed on a microscale balance and kept frozen in Greave's medium until cultured aerobically and anaerobically on nonselective (blood agar) and on selective media (MS agar, Rogosa agar and CFAT agar). Although approximately the same volume of dentin was collected for each sample, the weight was slightly different. On an average the weight of the carious dentin samples was 0.15 mg/ml and that of the non-carious samples 0.20 mg/ml (p<0.01). Total and selective bacterial counts were calculated as colony forming units (cfu) per mg dentin. Bacterial speciation was based on growth on the selective media, biochemical tests for *L. acidophilus* and *A. naeslundi* and simple or nested PCR for *S. mutans* and *S. sobrinus*.

Results: Samples 2 and samples 3 showed significantly (p<0.01) lower cfu/mg dentin counts on all culture media than did samples 1. Cfu/mg dentin of samples 3 were significantly (p<0.05) lower than those of samples 2, a difference most pronounced for anaerobic blood agar counts and CFAT counts. The Carisolv-2 gel inhibited the growth of all the cariogenic bacteria tested (*S. mutans, S. sobrinus, L. acidophilus, A. naeslundi*) with inhibition zones in the range of 16.5-35.0 mm and for *A. naeslundi* from 32.0 to 35.0 mm.

Conclusion: Treatment of carious lesions by the Carisolv-2 system significantly reduced the bacteria in the adjacent non-carious dentin as compared to non-carious dentin obtained from lesions treated by drilling. The gel is the antimicrobial agent of the Carisolv-2 system.

INTRODUCTION

Dental caries

Definitions

Caries (Latin, rottenness) is a gradual decay and disintegration of soft or bony tissue, or a tooth (Taber's Cyclopedic Medical Dictionary, 1997). Dental caries is a dynamic process, taking place in the microbial deposits (dental plaque on the tooth surfaces), which results in a disturbance of the equilibrium between tooth substance and the surrounding plaque fluid so that, over time, the net result is a loss of mineral from the tooth surface (Thylstrup and Fejerskov, 1994).

Epidemiology

Dental caries is one of the most common infectious diseases of mankind. It became a major health problem in most countries mainly because of its high prevalence. Its consequences upon oral and general health of individuals (pain, impairment of function, reduced quality of life) were one of the determinant factors for "Oral Health" to become a special unit in the World Health Organization (WHO), Geneva, in 1956. The goal for oral health in the year 2000 was to reduce decayed, missing or filled permanent teeth (DMFT) so that it would be no more than 3 DMFT per individual at 12 years of age. This goal was achieved by 68% of the countries (WHO Report, 2003). However, there is still a very unequal distribution of caries prevalence between countries, not very much related to their developmental stage, but more likely related to the individual socio-economical status.

Etiology

Dental caries is a multifactorial infectious disease that does not develop in the absence of dental plaque (dental biofilm). The principal (cariogenic) bacteria are transmissible and usually acquired in infancy. According to the Keyes' host-agent-environmental model (Fig. 1), the necessary causes of caries include the simultaneous presence of caries susceptible teeth, cariogenic microorganisms and suitable substrate in the form of dietary fermentable carbohydrates (Keyes, 1962). These three factors must be present for a

sufficient length of *time* for caries to develop. The cariogenic microorganisms, substrate and time are the cariogenic factors while saliva protects against caries.
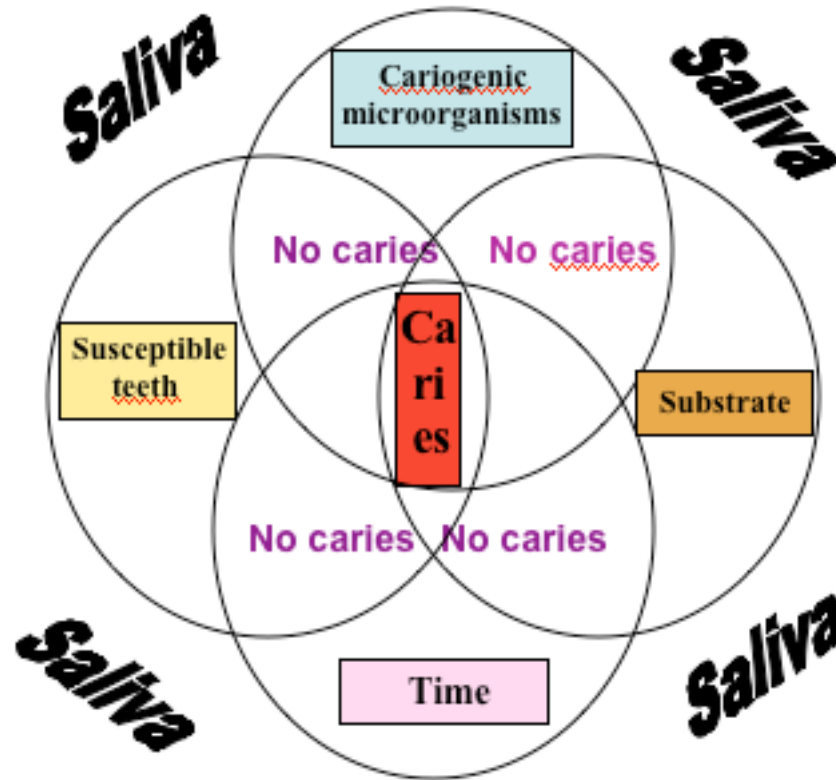
Fig. 1. Keyes' host-agent-environmental model for dental caries.

Cariogenesis

In the biofilm, the cariogenic bacteria produce acids from fermentable carbohydrates when available. The acids cause a pH drop. At about pH 5 (critical pH), demineralization of enamel starts. The pH values of top and bottom layers in cavities with small clinical openings and thick layers of decay, were lower than in cavities with thin layers and large clinical openings (Dirksen et al. 1963). When fluorideapatite replaces hydroxyapatite, the critical pH drops. Caries is a cyclic process with periods of demineralization (caries progress) followed by periods of remineralization (caries repair).

Dental caries may occur on any tooth surface where a microbial biofilm is allowed to develop and remain for a period of time. The presence of the biofilm does not necessarily

result in a caries lesion, but it is a necessary factor. If the biofilm is removed partially or totally, the mineral loss may be stopped or even reversed towards mineral gain.

Bacterial acids will dissolve the minerals and the collagen becomes exposed to bacterial acids and enzymes. A carious lesion in dentin can be described as a gradient of tissue destruction. The outer layer is infected, minerals are completely lost and the collagen is necrotic. Closer to the pulp the mineral content increases. As long as the collagen is embedded in minerals, acids and enzymes have little effect on it. The response of vital dentinoblasts to an advancing caries attack is reactive dentin deposition (secondary dentin) on the pulp walls. The dentinal tubules also become narrowed by intratubular mineral deposition. Sometimes this leads to complete occlusion of the tubules (sclerotic dentin). Production of secondary and sclerotic dentin should be kept in mind when treating caries (Bornstein and Ericson, 2001).

Dental plaque (dental biofilm)

There are approximately six billion microbes representing more than 500 established bacterial taxa in the oral cavity (Moore and Moore, 1994). Most of these organisms are commensals, but a part of them can become opportunistic pathogens that can cause oral or general diseases when disseminated.

Dental plaque is a microbial biofilm. The term biofilm is used to describe communities of microorganisms attached to a surface. The organisms are organized in a three-dimensional structure enclosed in a matrix derived from both the microbes themselves and the environment. The majority of all microorganisms live in biofilms while a small minority live in suspensions and are called planktonic microorganisms (Fejerskov and Kidd, 2003).

In humans bacteria adhere to the mucosal membranes or to the acquired enamel pellicle (glycoproteins) on the teeth. Most of the human species grow optimally at neutral pH (pH 7). If the host has a carbohydrates-rich diet, some oral bacterial species (acidogenic bacteria) will ferment the carbohydrates and produce acids. At the same time, the local pH will drop. Many bacteria cannot survive at pH lower than 5.5, but aciduric bacteria (i.e. the cariogenic bacteria) grow well (acidophilic) and are thus selected by the low pH (Kuramitsu and Richard, 2000).

Plaque hypotheses for dental caries

The non specific plaque hypothesis (Theilade and Theilade, 1976) considered dental diseases as the outcome of the overall activity of the total plaque microflora. The specific plaque hypothesis (Loesche, 1986) proposed that out of all the organisms in the plaque microflora, only a few species are actively involved in the disease. More recently, the ecological plaque hypothesis (Marsh 1991; Marsh 1994; Marsh 2003) (Fig.2), was introduced suggesting that disease is the result of a shift in the balance of the resident microflora driven by changes in local environmental conditions. For caries this means that repeated sugar intake, low salivary secretion and/or low salivary buffer capacity will lead to a maintained long pH period that will favor growth of acidogenic and aciduric microbial species. The ecological plaque hypothesis shows the lack of total specificity in the microbial etiology of caries and explains the pattern of bacterial successions observed in many clinical studies. It also shows the connection between the host (saliva flow, diet) and the microflora.
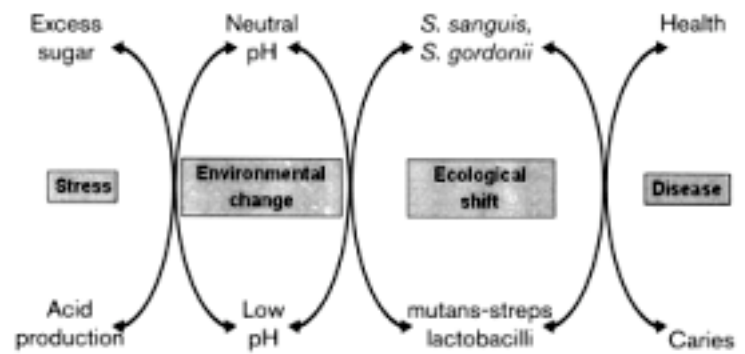
Fig. 2. The ecological plaque hypothesis for dental caries (Marsh, 2003).

Carious dental tissue

The carious tissue consists of demineralized, partially disrupted collagen fibers, together with other components of extracellular organic matrix of the dentin. Most of this matrix consists of proteins (many of them glycosylated). In addition, caries dentin contains bacteria and bacterial cell remanents, foodstuffs and other components absorbed from the oral cavity. As a consequence, caries has a heterogeneous composition. Its various components are glued together by the degraded and modified fibrous collagen type I, which

probably makes up the largest mass of carious tissue, since it constitutes over 90% of the organic matrix in dentin.

During the carious process, collagen is altered. Intermolecular cross links are disrupted. Non-collagenous proteins associated with collagen fibrils disappear. This could have more than one reason: they are substrate for bacteria, they are susceptible to degradation by bacterial exoenzymes, they are unstable at lower pH, and there is also some diffusion from the lesion - from soft carious dentin at least - to the oral cavity (Bornstein and Ericson, 2001).

Cariogenic bacteria

Mutans streptococci

Streptococci have been isolated from all sites of the mouth and they constitute a large proportion of the oral microflora. They have been divided into four main species (spp.) groups: the *mutans*-group, the *salivarius-* group, the *anginosus-* group, and the *mitis*-group. *S. mutans* was originally isolated from carious human teeth by Clarke in 1924. Little attention was paid to this species until the 1960s when it was demonstrated that caries could be experimentally induced and transmitted in animals artificially infected with strains resembling *S. mutans*. Its name derives from the fact that cells can lose their coccal morphology and often appear as short Gram-positive rods or as cocco-bacilli. The major species in humans are *S. mutans* and *S. sobrinus* with occasional isolation of *S. rattus* and *S. cricetus* (Kuramitsu and Richard, 2000).

Eight serotypes (a-h) were recognized based on the serological specificity of carbohydrate antigens located in the cell wall: *S. mutans, serotypes c, e, f; S. sobrinus, serotype d, g; S. cricetus, serotype a; S .rattus, serotype b; S. downei, serotype h; S. ferus* and *S. macacae* also belong to the same group.

The antigenic structure of mutans streptococci has been studied in detail for use in caries vaccine development or in serological typing schemes. They possess cell wall carbohydrate antigens, lipoteichoic acid, lipoproteins and cell wall or cell associated proteins. Antigen I/II (also named antigen B or P1) has generated considerable interest because of the inclusion in a possible sub-unit vaccine. This antigen may be involved in the initial

adherence of *S. mutans* to the tooth surface by interacting with components of the salivary pellicle.

Mutans streptococci synthesize extracellular polysaccharides (ECP) from glucose and fructose, obtained mainly by hydrolysis of sucrose, by means of glucosyltransferases (GTFs) and fructosyltransferase (FTF), respectively:

- soluble ECP: glucans and fructans that can be metabolized by other bacteria
- insoluble ECP: predominantly glucans that make a major contribution in plaque formation, can consolidate the attachment of bacteria in plaque and are also associated with cariogenicity (Marsh and Martin, 1999)

S. mutans possesses three GTFs - encoded by *gtfB*, *gtfC* and *gtfD* genes - which synthesize:

- α−1-3 polymers, encoded by *gtfB* and *gtfC*, which are insoluble glucans that contribute to plaque formation and are essential for caries initiation on smooth surfaces of teeth in animal models

- α−1-6 linked glucans, encoded by *gtfD*, that are water soluble and therefore not a *S. mutans* adhesin

S. mutans has a single FTF, encoded by the *ftf* gene, which catalyses the incorporation of the fructose component of sucrose into a fructan polymer that contains ß-2-1 linked fructose units. Fructans are not involved in adhesion and they do not remain for long in plaque. They act as extracellular carbohydrate storage compounds in plaque biofilms, being broken down to fructose by fructan hydrolases produced by a large range of oral bacteria.

S. sobrinus has been shown to have 4 GTFs activities and produce: a glucan composed mainly of α−1-3 linked glucose residues, 2 gene products which produce polymers with mixed α−1-3 and α−1-6 linked glucose molecules, and a linear glucan composed predominantly of α−1-6 molecules.

Mutans streptococci also synthesize intracellular polysaccharides which act as carbohydrate reserves, and can be converted to acid during periods when dietary sugars are not available. These bacteria produce acid very quickly from fermentable carbohydrate, and this contributes to their pathogenic potential in caries. Of equal significance is their ability to grow and survive under the acidic conditions they so generate (Marsh and Martin, 1999).

Actinomyces

Actinomyces spp. form a major portion of the dental plaque microflora, particularly at approximal sites and the gingival crevice. Animal studies reported in the early 1970s established that *Actinomyces* have a role in caries etiology, although a variety of later studies were unable to show a positive association for this genus in human caries etiology (Kuramitsu and Richard, 2000). *Actinomyces* cells appear as short Gram-positive rods, but are often pleomorphic in shape; some cells show a branching morphology, *A. naeslundi*, for example, is fimbriated and *A. israelli* filamentous. *A. naeslundi* is a single species but with two genospecies that can be differentiated only serologically.

All *Actinomyces* spp. ferment glucose to a characteristic pattern of metabolic end product (succinic, acetic and lactic acids).

Lactobacilli

Lactobacilli are frequently isolated from the oral cavity though they represent only about 1% of the total cultivable microflora. But their proportion and prevalence increase in advanced caries of the enamel and root surface. They produce either lactate or lactate and acetate from glucose. The most commonly detected species in the oral cavity are *L. casei* and *L acidophilus*. *L. acidophilus* has been sub-divided into *L. acidophilus sensu stricto*, *L. crispatus* and *L. gasseri*. *L. casei* – group includes several distinct species, for example: *L. rhamnosus*, *L. paracasei*. The lactobacilli are highly acidogenic bacteria (Marsh and Martin, 1999).

Bacterial shift during caries progress

Mutans streptococci are mainly implicated in the initiation of enamel caries, and they are only rarely the predominant species isolated from carious dentin of deeper lesions. The lactobacilli are associated mainly with carious dentin and the advancing front of carious lesions. The composition of the cariogenic microflora becomes more complex as the lesions progress, and obligate anaerobes, mainly Gram-positive rods (*Lactobacillus, Actinomyces, Bifidobacterium, Propionibacterium, Eubacterium, Rothia*) become the predominant cultivable organisms in teeth without endodontic infections (Azrak et al., 2004).

Bacteria of enamel, dentin and root caries

The strongest relation between plaque levels of mutans streptococci and caries sites was found in fissures. It seems that carious fissures also present dental plaque with high levels of mutans streptococci, whereas caries-free fissures have no detectable mutans streptococci. But, there are also studies that found no mutans streptococci in carious fissures or high levels in caries free fissures (Fejerskov and Kidd, 2003). Mutans streptococci and lactobacilli were found more frequently or in higher proportions on carious root surfaces than on the healthy ones. In addition, there were found species of *Actinomyces*, non-mutans streptococci, *Bifidobacterium*, *Candida*, enterococci, *Veillonella* and anaerobic Gram negative species. Lesions with high mineral loss are dominated by a few acidogenic species (*Actinomyces* spp. or combination of mutans streptococci and lactobacilli). Lesions with a small loss of mineral substance are associated with a more complex flora (mutans streptococci, *Actinomyces* spp., *Lactobacillus* spp., *Veionella* spp. etc) (Fejerskov and Kidd, 2003). *A. naeslundi* has been implicated in root surface caries but its role is still under debate (Marsh and Martin, 1999).

Residual bacteria in filled cavities

Studies have examined bacteria left in cavities after temporary fillings. Calcium hydroxide and zinc oxide and eugenol proved to be more effective in reducing the bacterial growth than amalgam (King et al., 1965; Bjørndal et al., 1997). The nutrients diminish and no microbial layer persists. The bacteria probably survive by taking nutrients from tissue fluid in the pulp via dentinal tubules or perhaps from the tissue in which they remain. After a time interval, a re-entry found the dentin to be darker and harder as well as drier (King et al., 1965). An explanation for these changes could be re mineralization (Fejerskov and Kidd, 2003).

The question now arises is: if infected dentin may be safely left in the cavity - up to 10^2 cfu/ml (Kidd et al., 1993; Bjørndal et al., 1997), could it then be possible to arrest the caries process by simply sealing the cavity in the tooth? Considering the huge improvement of composites in the sealing of the cavities, this might be an option of the future. Some studies show that in time the small lesions seem to regress radiographically

and in two years follow-ups no clinical caries was reported, indicating that the carious process had been arrested (Handelman et al., 1986).

Dental caries treatment

While the initial enamel lesions can be treated only by removing the biofilm during tooth brushing and applying fluorides, the cavitated lesions need to be approached by operative methods. Until a few years ago, the only effective method for removing carious dentin was the high- or low- speed bur. This traditional method requires sacrifices of healthy dental tissues, might cause injuries of the pulp and shorten the tooth survival. Because of the development of new filling materials (composites), there is no need for a certain shape of the prepared cavity. Composites have a chemical and micromechanical adhesion. Less sacrifice of healthy dentin is necessary than when using traditional filling materials (e.g. amalgam) and strictly following Black's principles for cavity preparation. Also, the use of fluorides has reduced the need for extension for prevention, as another Black's principle required. The only invasive procedure is drilling. In order to support minimally-invasive procedures, a new chemo-mechanical method named Carisolv® (see page 17) was developed for caries tissue removal.

More and more it is stressed today, that a conservative treatment should be performed, removing as little as possible of the tooth tissue (Fejerskov and Kidd, 2003).

Today, the most used approach is to remove the soft tissue of carious lesions with an excavator or bur. The most accepted procedure is the one that suggests to stop excavation when hard dentin, even if stained, is reached. A special case is that of dentin over the pulpal surface that should be "reasonably hard" (Fejerskov and Kidd, 2003). However, the new chemo-mechanical methods developed provide now a new and more conservative perspective upon caries treatment.

Conventional (traditional) treatment methods

Drilling

One of the most common problems in clinical dentistry is how to minimise the risk of opening the pulp chamber, especially when using burs. It is still a debate whether caries excavations should be performed in one step or step-wise in order to give a chance to the

physiological reactions in the pulpo-dentinal organ. There are also recommendations to treat the cavity floor with calcium hydroxide or zinc oxide eugenol. The temporary sealing intervals vary between four to eight weeks and two to six months. Studies have shown that teeth with *chronically inflamed pulp* and infected dentinal tubules on the cavity floor were associated with cultivated streptococci and lactobacilli from the dentin samples. In cases of histologically assessed *healthy pulps* with infected dentinal tubules and a remaining dentin layer of 0.4 mm, *Actinomyces* was the dominantly cultivated microorganism (Heinrich-Weltzien and Kneist, 2001).

Minimal-invasive methods

Hand excavation

Excavation is performed with a sharp excavator. It uses the Atraumatic Restorative Technique (ART) that includes the removal of the soft dentin with the excavator and restoration using adhesive filling materials, especially glass ionomers (Banerjee et al., 2000a). The main problem seems to be the issue of leaving infected dentin behind, the pain that sometimes is felt by the patient and non-discrimination between the healthy and infected dentin.

Air-abrasive techniques (kinetic cavity preparation)

It is similar to sandblasting procedures, using particles (e.g. aluminium oxide) propelled by an air stream. It was meant to be used for fissure treatment and can abrade hard tissues, but it was not shown to be very effective in dentin compared to other methods (Banerjee et al., 2000a).

Sono-abrasion

This method uses a diamond coated tip that oscillates and can be used for micro preparations and standardized inlay preparations. It is very precise but it seems to be less suited for effective removal of dentin caries (Banerjee et al., 2000a).

Lasers

This system is effective for enamel and healthy dentin ablation, but not much data was reported about selective caries removal. The energy from the laser beam is transferred to the dental tissue and absorbed by water molecules that evaporate instantaneously and break off pieces from the surface (ablation) (Bornstein and Ericson, 2001).

Enzymes

Although no clinical data is available about the use of enzymes, the method was able *in vitro* to solubilize carious dentin using an enzyme preparation containing proteases (pronase). However the time needed was very long, four consecutive 24 hour applications (Bornstein and Ericson, 2001).

HealOzone

The ozone delivery system is a portable apparatus for the treatment of caries

with an ozone generator that delivers ozone at a concentration of 2,100 ppm ± 10%. The rapid inactivation of microorganisms is one of the outstanding characteristics of ozone. This system has shown promising results, especially in initial caries but further studies need to be performed to assess its action upon the complex carious flora (Baysan and Lynch, 2004**).**

Chemo-mechanical treatment

Carisolv® is a two-component gel designed to soften the altered dentin and make possible its gentle removal with specially designed scraping instruments. The idea of developing a chemo-mechanical treatment for caries removal has originated in the 70s. The goal was to create a chemical preparation to act selectively on denatured collagen. Habib et al. (1975) and Kronman et al. (1977) found that sodium hypochlorite was useful. But it was shown that NaOCl was dissolving not only necrotic tissue but also sound dentin (Hand et al., 1978).

The Carisolv® system
Development, composition and principle of action

Fig. 3 shows the development of the Carisolv system.
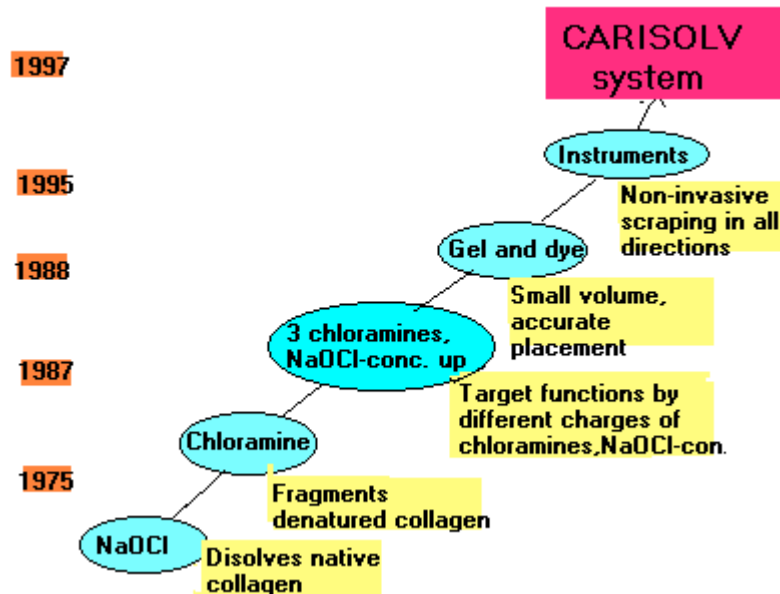
Fig. 3. Development of the Carisolv system by Dan Ericson and Rolf Bornstein, Malmø, Sweden (Bornstein and Ericson, 2001).

 The action of chloramines was explored in an early system for chemical caries removal (Caridex, USA) (Romand-Roeloffs et al., 1991). This system was using NMAB (N-monochloro-DL-2-aminobutyric acid). Its action seems to involve the chlorination of partially degraded collagen and the conversion of hydroxyproline to pyrrole-2-carboxylic acid, which initiates disruption of altered collagen fibres in carious dentin. NMAB allowed in this way the carious dentin to be removed more easily, by separating it from the healthy dentin underneath, using a modified hypodermic needle and an irrigating system. This early system had many limitations, such as difficult clinical handling, large volume of solution needed, short shelf-life of opened packages and time required for complete caries removal. It also included a large heated reservoir and a noisy pump for solution delivery (Heinrich-Weltzien and Kneist, 2001)

During the 1970s, adhesive filling materials were not in common use. Restorative procedures included mechanical retention and the need for cutting healthy dentin, so these circumstances limited also the use of the chemo-mechanical method.

Strid and Hedward (1987) thought of generating a group of more efficient chloramines, by using aminoacids with different charges (positive, negative and neutral), so that the molecules could electrostatically or hydrophobically interact with different parts of denatured collagen. The following aminoacids are used as homing devices for active chlorine: glutamic acid, leucine and lysine. At the same time, Bornstein and Ericson (2001) tried to improve the clinical efficacy. The solution was changed to a more viscous gel that makes it easier to handle, requires a smaller amount of material and eliminates the need for a pump (Bornstein and Ericson, 2001).

The sodium hypochlorite concentration was increased five times compared to the NMAB system. This made the system more efficient, so instead of cutting in dentin, a more gentle removal is possible because of a better softening of dentin. A red dye (erythrosine) was added to make the gel easier distinguishable from the tooth surfaces. Also, a set of new instruments was designed to scrape in many working directions, unlike the classical excavators that can act in only one direction. The whole system was named Carisolv® (referred to as Carisolv-1 below) and it was launched in 1997 by MediTeam Dental AB Sweden.

The first version of Carisolv (Carisolv-1)

Details about Carisolv-1 has been published (Bornstein and Ericson, 2001; Lager et al., 2003). When mixing the two Carisolv components (two syringes) prior to treatment, at pH 11, stable monochlorinated forms of these aminoacids are formed. The chlorine atom of hypochlorite is transferred to the amino group of each amino acid and in this way it is made less reactive and less aggressive to healthy tissue. The alkaline pH ensures suppression of formation of more reactive chlorine species like dichlorinated amines and hypochlorous acid. Besides, in a reducing environment of an alkaline solution, chlorination rather than oxidation of an organic molecule is favored (the oxidative property of hypochlorite is suppressed). By including the three chloroaminoacids with different side chain properties, positively and negatively charged and hydrophobic, it is

ensured that they will electrostatically attract all three possible protein patches, not only collagen, but also all proteins and large organic molecules. It is still not certain how the disruptive power of chlorine is exerted in the target tissue, but it probably occurs at non covalent bonds, like hydrophobic or van der Waals interactions. These, although they are individually weak, are present in large numbers.

The specificity towards proteins introduced by the amino acid chlorination gives the protection potential for the healthy dentin, which is largely non-proteinaceous and has as its major constituent the mineral hydroxyapatite. Also, the high pH stabilizes the mineral structure by decreasing its solubility (this is favored at low pH) (Heinrich-Weltzien and Kneist, 2001).

The second version of Carisolv (Carisolv-2)

In order to overcome the difficulties met when working with Carisolv-1 (long time, lack of efficiency in some cases), many trials have been performed resulting in Carisolv-2. In this version, the concentration of NaOCl has been increased in order to increase the efficacy (i.e. antibacterial and collagen-solving effect) of the gel. The red dye was abandoned because it was considered a visual obstacle in assessing the status of clean, healthy dentin (Azrak et al., 2004; Fure and Lingstrom, 2004).

Biocompatibility

Histological evaluation of pulp exposed to Carisolv (the study does not indicate which version) and sterile saline (control) from class V cavities, showed structural integrity after one month in both cases. However, after one week a slight inflammation was observed in both groups; the difference was that the control group presented localized haemorrhage, while no haemorrhage was observed in the test group. The conclusion was that Carisolv is biocompatible with human pulp tissue and may have a haemostatic effect (Bulut et al., 2004). Another study tried to estimate the cytotoxic effect of Carisolv-1 on the mouse mammary carcinoma cell line FM3A. Cell growth was significantly reduced after 20 min application of Carisolv-1 as compared to the control and to the 1- minute treatment groups. No significant differences were found in cell viability between the study groups (Sepet et al., 2004).

Most effects of Carisolv-1 on the oral mucosa are harmless. Some studies, however, have indicated a tendency towards an increased inflammatory reaction 24 hours after application directly on the mucous membrane. The reaction was not strong and 48 hours after the application the number of cells was similar to that found in the control tissue. Twenty-four and 48 hours after application, there was no discomfort clinically (Young, 2001).

Efficiency

Some studies found that Carisolv-1 did not remove completely the carious dentin from the enamel-dentin junction (Cederlund et al., 1999), so that a proper selection of cases should be done, remembering that a large access to the cavity is necessary. Clinical studies have confirmed the laboratory findings and Carisolv-1 is considered effective for root and coronal caries (Ericson et al., 1999). The time required to remove caries with Carisolv-1 is longer compared to rotating instruments. Studies have shown the time needed for a conventional treatment for caries with a bur to be 4.4±2.2 min, using Carisolv-1 10.4±6.1 min were required. But for root caries the time difference was approximately 1 minute, which is not considered a clinically relevant difference (Ericson et al., 1999; Fure et al., 2000). Using excavators, the treatment took approximately the same amount of time, but Carisolv-1 was significantly less painful. All patients participating in studies with Carisolv-1 had a favourable perception.

The only study with Carisolv-2, reported so far, showed that compared with NaOCl and $Ca(OH)_2$, Carisolv-2 reduced caries significantly better when assessing remaining caries with light-microscope (Dammaschke et al., 2004).

Another recent study (Azrak et al., 2004) which does not specify the version of Carisolv used, showed in children that Carisolv produced a significant reduction of the total bacterial growth and lactobacilli and was considered a good alternative to drilling.

Advantages

The Carisolv method is gentle, minimally-invasive and has a high degree of acceptance among patients, and it eliminates pain and the need for anaesthesia. It removes only

affected dentin, so there is no sacrifice of healthy tissue. It is a self - limiting method. There seems to be no effect on healthy dentin.

Recommendations and restrictions for use

The Carisolv system is highly recommended for paediatric treatment and dental care for mentally disabled patients. It has been proved to be very efficient in removing root caries and deep, sensitive caries eliminating the need for anaesthesia. Still, it is not a universal treatment. There is a need for a very good access to the altered dentin, because the Carisolv gel is not effective in enamel. That is why, proper selection of cases or sometimes, a combined treatment is needed (Cederlund et al., 1999). Some studies showed that the bacteria from the enamel dentin-junction could not be eliminated completely. There is a need for a very good access to the altered dentin. The patients may consider Carisolv treatment expensive and the treatment time long. New versions of Carisolv have been tested to find out the most efficient combination and concentration of its components.

RATIONALE OF THE STUDY

Carisolv-1 gel has demonstrated significant antimicrobial effect *in vivo,* reducing total bacterial counts, streptococci and lactobacilli (Lager et al., 2003). In agar diffusion tests, the Carisolv-1 gel produced inhibition zones of 13-15 mm for *A. naeslundi*, *S. mutans*, *S. sobrinus* and *L. casei* (Kneist and Heinrich-Weltzein, 2001). No information about the antimicrobial effect of Carisolv-2 has yet been published. This fact prompted my master's research project.

OWN HYPOTHESIS

Based on information about antibacterial effect of the Carisolv-1 system, I formulated my work hypothesis that the Carisolv-2 system has antibacterial effect and that this effect is due to the gel included in the system. To test my hypothesis, I set up a null hypothesis that there is no significant bacterial difference between non-carious dentin from caries lesions that have been treated by drilling or by the Carisolv-2 system.

SPECIFIC AIMS

1. To assess *in vitro* the *in situ* antimicrobial effect of Carisolv-2 gel on the bacteria in carious lesions.

2. To compare the type and quantity of bacteria found in the non-carious dentin from the bottom of the cavities after treatment with conventional drilling with the bacteria found in the dentin after treatment with Carisolv-2 gel.

3. To evaluate the antimicrobial effect of Carisolv-2 gel on selected cariogenic bacteria by estimating the inhibition areas determined in agar gel.

MATERIALS

Extracted teeth

Twenty-four human permanent teeth (16 molars, six premolars and two canines) with primary carious lesions (class I, class II and class III) were collected from patients (age: 18-52 years) treated at the Surgery Department, the University Hospital of Stomatology "Prof. Dr. Dan-Theodorescu" Bucharest, Romania. Reasons for extraction were periodontal disease, orthodontic problems and wisdom molars. Only teeth with lesions that did not involve the pulp were included in the study. An informed consent that the extracted teeth would be used in this study was signed by each patient.

Dentin samples

Samples 1: Carious dentin obtained before treatment.

Samples 2: Hard dentin sampled from the bottom of the carious lesions from which the carious dentin had been removed by drilling (see below).

Samples 3: Hard dentin from the bottom of the carious lesions collected after application of Carisolv-2 gel.

Carisolv-2 gel (Mediteam, Sweden)

The gel is prepared by mixing the contents of two syringes and should be used within 20 min. One of the syringes contains glutamic acid, leucine, lysine, NaCl, carboxymethylcellulose, water, and NaOH at pH 11, and the other 0.5% NaOCl and alanine aminotransferase.

METHODS

Assessment of dentin status

There are not any perfect clinical criteria to determine whether a cavity is caries-free or not. The methods in use today, such as dentin hardness on probing, colour, structure and moisture of dentin (Banerjee et al., 2000b), are very subjective.

A newly developed diode-laser caries detector (DIAGNOdent KaVo, Germany) (Fig. 4) was used in this study to determine dentin status thus distinguishing between carious and sound dentin. The fluorescent light is measured and its intensity is an indication of the presence of the caries lesion. This intensity is displayed as a number (from 0 to 99) (Lussi et al., 1999). Each measurement was performed twice by two different operators (RID and a Romanian colleague) and the mean value was utilized.

Fig. 4. KaVo DIAGNOdent (KaVo Germany).

Collection of dentin samples

Each tooth was handled aseptically and split in two halves along its long axis through the middle of the carious lesion using a water-cooled sterile diamond disk (Fig. 5). Before tooth splitting, a sample of the carious dentin (sample 1) was taken. After splitting and

treating each half of the caries lesion with drilling or Carisolv, samples of hard dentin (samples 2 and 3) were obtained.
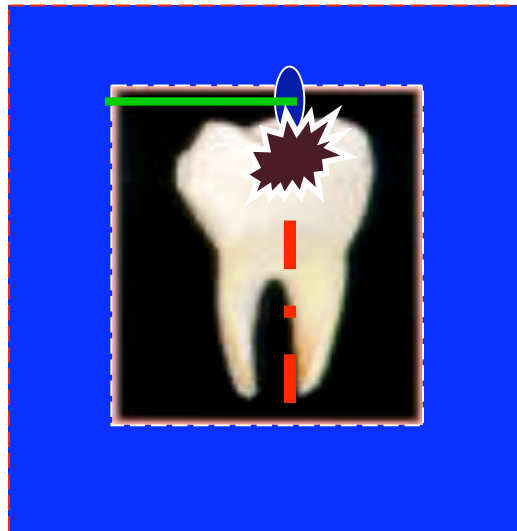
Fig. 5. Tooth splitting.

All samples were collected by means of sterile round burs no. 18 (Hager & Meisinger, Germany) at low speed. In cases with narrow entrance to the carious lesion, high-speed hand piece with sterile burs were first used to improve the access. When the bur was fully covered by carious dentin (sample 1) or dentin powder (samples 2 and 3), it was placed in a vial containing a Greave's medium and vortexed for 30 sec (Heidolph Reax). Then, the bur was removed using sterile tweezers (Ratledge et al., 2001).

Samples 1 were obtained after removing the superficial layer of debris with an excavator.

Samples 2 were taken after all carious dentin had been removed using conventional burs smaller than those used for collection of samples. To determine that sound dentin with the same hardness had been reach at the bottom of the cavities, a dental explorer and the DIAGNOdent were used (Lussi and Francescut, 2003).

Samples 3 were taken after the Carisolv treatment and in the same way as samples 2.

Fig. 6 shows the application of the Carisolv®- 2 gel which was prepared and applied according to the manufacturer's instruction. First, the two syringes were removed from the refrigerator one hour before mixing their contents. Then, one drop of the gel was applied with the star instrument and left in the cavity for 30 sec before excavation of the

softened carious dentin. The special designed instruments were further used for scrapping the cavity (Ericson et al., 1999). Depending on the effect, the gel was applied 3-5 times until healthy dentin was reached as indicated by KaVo DIAGNOdent readings below 12 (Lussi and Francescut, 2003) and observation that the gel no longer turned cloudy. The gel was removed with a sterile cotton pellet soaked in sterile saline and then the cavity was dried with sterile cotton pellets. After careful inspection of the cavity for complete caries removal, samples 3 were taken.
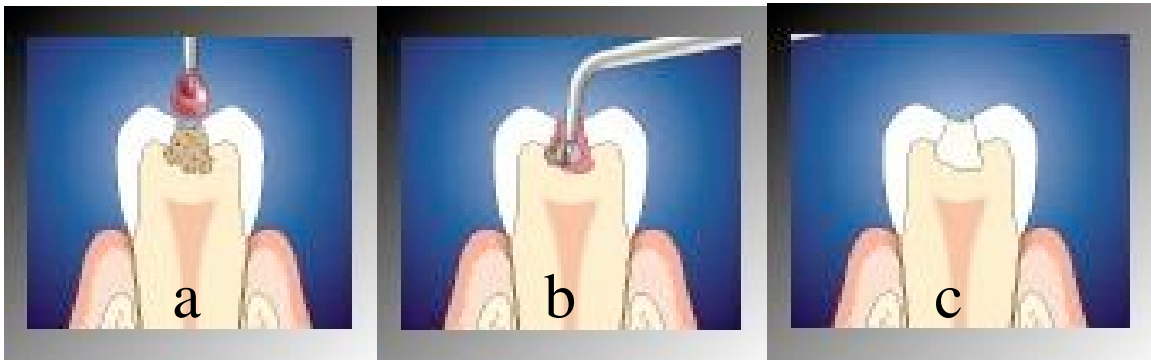
Fig. 6. The Carisolv®- 2 method: a) application of gel; b) removing carious dentin by scraping; c) clean cavity.

Weight of the samples

In order to test the accuracy of the sampling, the weight of the dentin samples was determined and compared. The procedure was as follows: 1) an empty sterile 1.5 ml tube was first weighed on a balance (Mettler, Spoerhase Ag. Giesen, Germany), 2) an empty bur in a tube was weighed and served as a standard, 3) the burs with carious dentin (samples 1) and 4) the burs with healthy dentin (samples 2 and 3) were put in a 1.5 ml tube and weighed. The weight of the tube and the drill was subtracted from the total measurements 3) and 4) to get the weight of the dentin. The mean weight obtained with the burs with carious dentin was compared with the mean weight obtained from the burs with healthy dentin. Subtracting these two values, the difference in the dentin weight between the healthy samples and the carious samples was obtained.

Storage of dentin samples

Pilot study

A pilot study was carried out at the Laboratory of Oral Microbiology, Faculty of Dentistry, University of Bergen before the extracted teeth (see page 18) were sampled to establish a preservation method for the dentin samples (samples 1-3 above) that would keep as much bacteria as possible alive for at least eight weeks.

Suspensions (1ml) of *S. mutans* (ATCC 27607), *S. sobrinus* (ATCC 25735), *L. acidophilus* (CCUG 5917), and *A. naeslundi* (X 600) were prepared using the McFarland standard 1 (approximate bacterial concentration 10^8/ml); respectively 2μl of the McFarland 1, 2μl and 20μl of the diluted McFarland 1 suspension (10^5/ml) were transferred to the following 1.8 ml preservation media: Greave's medium (see appendix A: preservation media), skim milk (see appendix A), reduced transport fluid (RTF) (Syed and Loesche, 1972), and VMG II (Møller, 1966). The bacterial concentrations obtained were: 10^5, 10^3 and 10^2 cfu/ml). Also, carious dentin samples from four extracted teeth obtained from the Faculty of Dentistry, University of Bergen were stored in the same media. Storage at –20° C and –70° C and anaerobically at ambient temperature was used.

After four and eight weeks, 1) the frozen samples were thawed, plated and the number of surviving bacteria (cfu/ml) calculated, and 2) the number of surviving bacteria in the samples stored anaerobically were calculated. The results showed that none or few bacteria survived in room temperature, in RTF and VMG II. The recovery of bacteria from skim milk varied while that of Greave's medium was the highest and most constant one. Samples stored at –20 C° for four weeks in the Greave's medium showed no significant difference compared to the initial counts. However, after eight weeks there was a significant decrease (p = 0.027). At –70° C there was no such decrease. Based on these results, storage of dentin samples in Greave's medium at –70° C was chosen.

The chosen method

Immediately after collection, samples 1-3 were transferred to plastic vials each containing 1.8 ml Greave's medium and stored for four to eight weeks at –70 C° until transportation.

Transportation of samples

The deep-frozen samples were transferred to a box containing dry ice (solid carbon dioxide) and transported the same day to the Laboratory of Oral Microbiology, in Bergen.

Identification of species and quantification

Microbiological methods

Cultivation

After thawing, the samples were diluted in TE buffer (10 mm Tris-HCl, 1.0 mm EDTA, pH 7.6). Undiluted or diluted samples (10 μl) were plated in triplicate for:

Total bacterial counts on non-selective media:

- Aerobic blood agar (Columbia agar, Acumedia, USA)

- Anaerobic blood agar (Fastidious Anaerobe Agar, Lab M, UK)

Identification of bacteria using selective media (for composition, see Appendix B):

- Rogosa lactobacilli selective agar (SL-agar; DIFCO 0480-01-8)

- Mitis salivarius agar (MS-agar; DIFCO 2298-10)

- Cadmium fluoride acriflavin tellurite (CFAT) agar

These selective growth media were used to identify lactobacilli, oral streptococci and *Actinomyces* spp, respectively. The samples were diluted as appropriate to obtain no more than 300 cfu/plate. The average median cfu/mg dentin sample obtained from the triplicates was used in the statistical analysis.

Figs. 7-11 show the growth on blood agar and on the selective media.
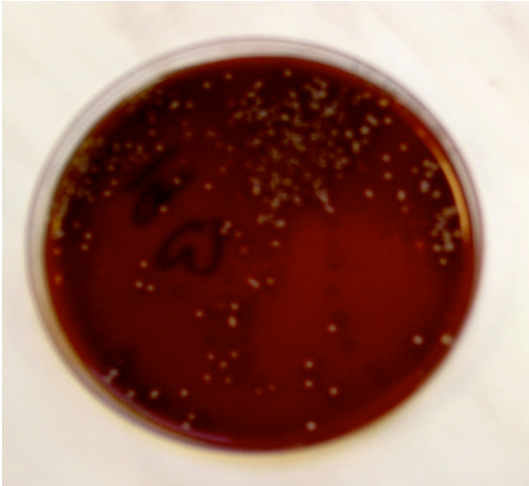
Fig. 7. Growth on aerobic blood agar.
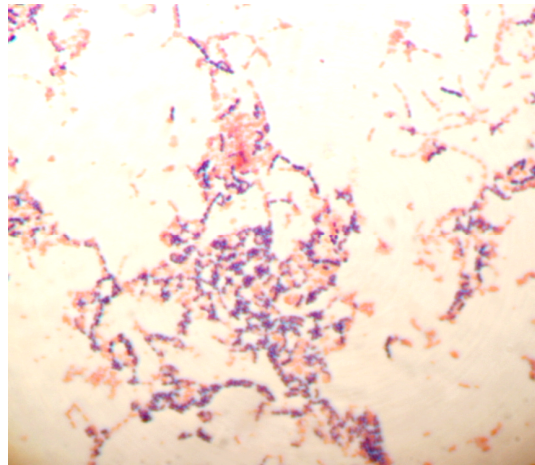
Fig. 8. Gram-stained smear of mixed flora on aerobic blood agar.
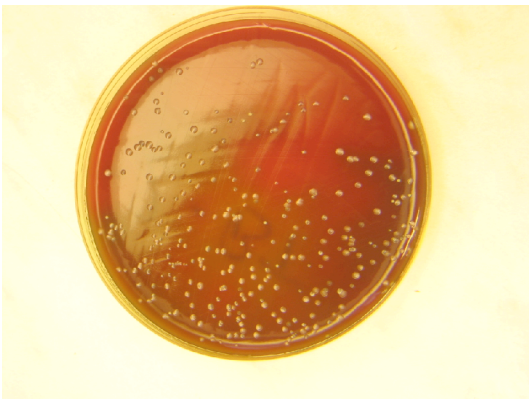
Fig. 9. Growth on anaerobic blood agar.
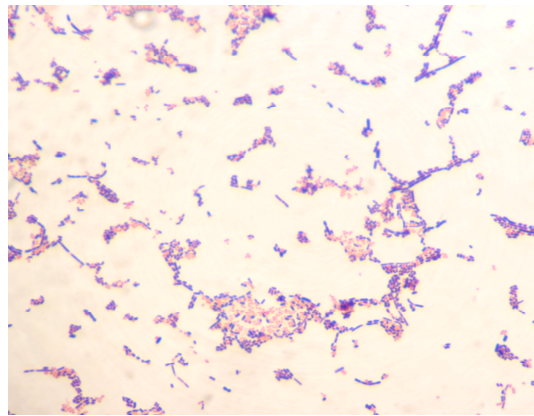
Fig. 10. Gram-stained smear of mixed flora.
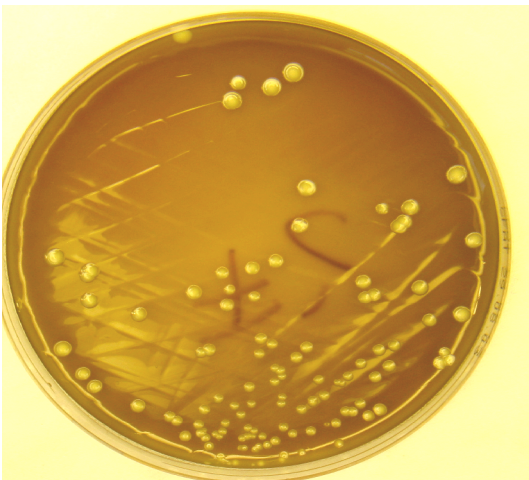
Fig. 11. Growth on CFAT agar.
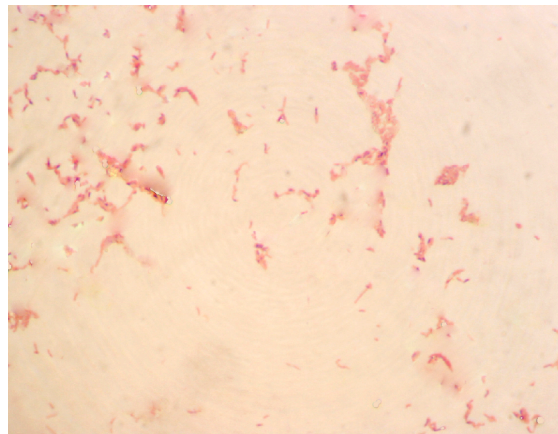
Fig. 12. Gram-stained smear of growth from CFAT agar.

Fig. 13. Rogosa agar growth.
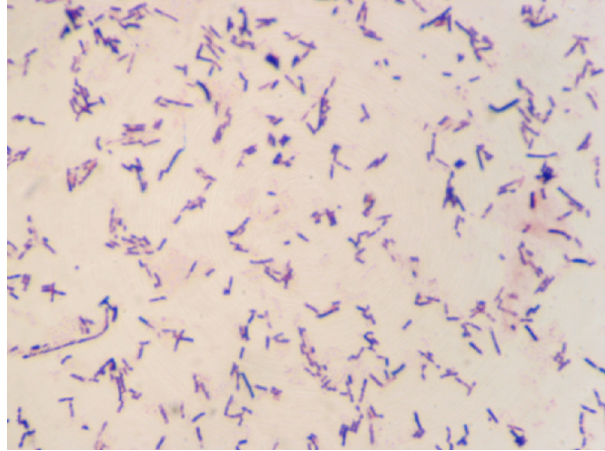
Fig. 14. Gram-stained smear of growth on Rogosa agar.
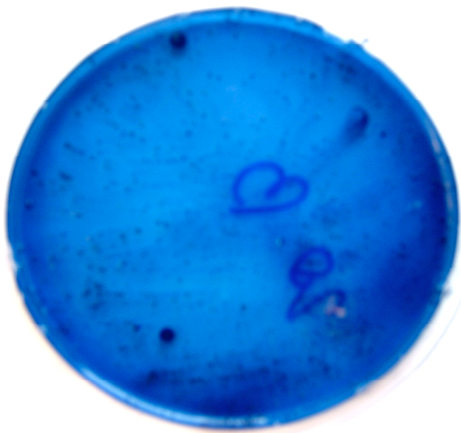
Fig. 14. Growth on MS agar.
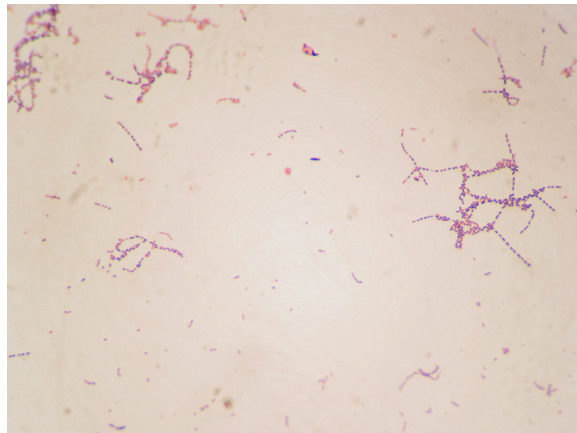
Fig. 15. Gram-stained smear of growth on MS agar.

Gram-stained smears

Gram-stained smears were prepared from single colonies with different morphology growing on the agar plates. The smears were used for bacterial identification and to verify pure cultures to be used in the biochemical tests (see page 31).

Oral streptococci

Cultivation aspects - Streptococci grow as small (1-2 mm in diameter), discoid grey colonies on sheep blood agar where they may exhibit α- or β-haemolysis, or they may be non-haemolytic. Colonies of strains that produce ECP are easily distinguishable from those of ECP non-producing strains.

Microscopic aspect - Streptococci are spherical gram-positive bacteria occurring in pairs or chains (Fig. 15). The members of the chain can have a diplococcal appearance and rod- like forms are occasionally seen. The lengths of the chains vary widely and are conditioned by environmental factors. Though they are gram-positive bacteria, as a culture ages and bacteria die, they lose their gram-positive color and appear gram-negative. This color shift can occur after overnight incubation.

Lactobacilli

Cultivation aspects - Lactobacilli form large white colonies on Rogosa agar (Fig. 13). Their metabolism is fermentative; some species are aerotolerant and may utilize oxygen through the enzyme flavoprotein oxidase, while others, like oral lactobacilli, are strictly anaerobic. Their growth optimum is at pH 5.5-5.8.

Microscopic aspect - Lactobacilli are gram-positive and vary in morphology from long, slender rods to short cocco-bacilli which frequently form chains (Fig. 14).

Actinomyces

Cultivation aspects –Medium-sized white, irregular colonies with a slow growth (two-five days) (Fig. 11). They are facultative anaerobic bacteria.

Microscopic aspect – Polymorphic gram-positive bacilli that are filaments, branched, non-branched, straight or curved, with different lengths, sometimes with cocco-bacilli shape (Fig. 12). Morphology alone cannot distinguish between *A. naeslundi* and *A. viscosus*.

Biochemical methods

Rapid ID 32 A (BioMerieux)

This is a standardized identification system for anaerobes with results available in four hours. It uses 29 miniaturized enzymatic tests and a database. The reactions obtained are coded into a numerical profile. The numbers thus obtained by summing up each three digit groups from the result sheet should correspond to a certain bacterial strain. Reading and interpretation can be carried out manually (as was done in this study) or automatically using an identification software (see appendix C for principle). Only pure bacterial cultures were used for this test.

The test was used to identify bacteria on CFAT. Three colonies were randomly picked from each such sample and re-plated on three different anaerobic blood agar plates which were incubated at 37° C for 24 hours. The next day, the growth on the blood agar plates was harvested and a suspension with a turbidity corresponding to McFarland 4 (1200 x 10^6 cells) was prepared. This suspension (55 μl) was transferred by pipette to each cupule of the Rapid ID 32 A strip. Then the lid was put back in place to cover the strip which was incubated aerobically for four hours at 37° C. The necessary reagents were added and finally he reading was performed within 10 min, according to the manufacturer's instructions.
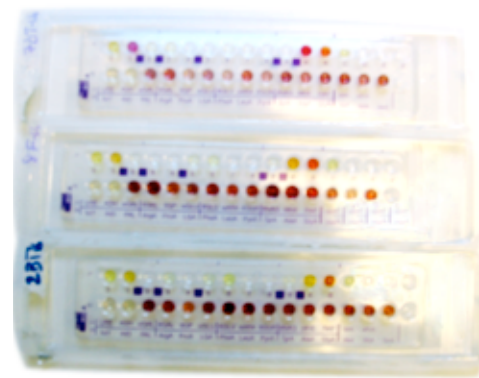
a)   b)

Fig. 17. Biochemical test strips for identification of a) *L. acidophilus* and b) *A. naeslundi*.

Rapid ID 32 STREP

It is a standardized system for the rapid identification of streptococci, enterococci, and the most related organisms. The test uses 32 miniaturized enzymatic tests and a specific database (for principle, see appendix C). In brief, in order to get pure cultures of the streptococci, isolated colonies were re-plated on blood agar after Gram-smear identification of the growth. The blood agar plates were incubated aerobically for 24 hours at 37° C. Suspension corresponding to the McFarland 4 standard was prepared and 55 μl were added in the cupules. After four hours of aerobic incubation at 37° C the reagents were added and the reading was performed within 5-10 min according to the manufacturer's instructions.

Because of problems regarding identification of *S. mutans* and *S. sobrinus*, we decided to use PCR for this identification.

Polymerase chain reaction (PCR)

Traditional methods for the identification of some bacterial species can be time consuming and often necessitates the isolation of pure cultures before. Advances in molecular biology have allowed the identification of bacterial species by virtue of the unique nature of the DNA of a species.

This technique, the polymerase chain reaction (PCR), was devised (Mullis 1990) and it has revolutionized molecular genetics by making possible a whole new approach for studying and analyzing genes. It enabled us to produce enormous numbers of copies of a specified DNA sequence without cloning (Watson et al., 1992).

DNA replication has some specific features that are exploited in the PCR process (see appendix D for PCR principle).

PCR was used in this study to identify specific species of streptococci (*S. mutans* and *S. sobrinus*), because the biochemical tests gave unsatisfactory results.

It is not necessary to isolate the sequence to be amplified because it is defined by the primers. Usually, little amount of DNA is needed for a PCR reaction, less than 1 μg of the total genomic DNA. It is not required to be a very pure DNA, for some bacteria it is enough just to release the DNA by boiling (Watson et al., 1992).

However, in this study, we tried several times to use the DNA from boiled bacterial suspension or extracted with a simple kit (Instagene kit) and this was good enough when using universal primers for streptococci, but not when using specific primers, especially for *S. mutans*.

The **electrophoresis on agar gel** is performed afterwards to evaluate the results of the PCR. The 1% agarose gel is used (1 g agar powder in 100 ml 1xTAE buffer).

DNA extraction

Pilot study

Boiling

This method was reported to be successful for DNA extraction from streptococci in a previous study (Oho et al., 2000). Aliquots (0.5-1.5 ml) of the Greave's samples were boiled at 100 °C for 10 min and then centrifuged 3 min at 12,000 rpm. The supernatant was used for species specific PCR and nested PCR (see below). No bands (PCR) or only few bands (nested PCR) were obtained even if different PCR cycles and different species-specific (*S. mutans* and *S. sobrinus*) primers were tried. With universal streptococci primers, however, the PCR results were acceptable.

InstaGene™ Matrix **(**Bio-Rad Laboratories, USA)

This method was reported to be successful for DNA extraction from streptococci in a previous study (Sato et al., 2003).

According to the manufacturer's instructions, cell lysis by boiling is sufficient because the matrix absorbs cell lysis products that interfere with the PCR amplification process.

I performed the following steps:

- an isolated bacterial colony was picked and suspended it in 1 ml autoclaved water in a micro-centrifuge tube.

- the tube was centrifuged for 1 min at 12,000 rpm. and the supernatant was removed

- 200 μl of InstaGene matrix was added to the pellet and incubated at 56° C for 30 min

- before use, the InstaGene matrix was mixed at a moderate speed on a magnetic stirrer to maintain it in suspension

- then the tube was vortexed at high speed for 10 sec and afterwards placed in a boiling water bath for 8 min

- the tube was vortexed again at high speed for 10 sec and then spinned at 12,000 rpm for 3 min

- 20 μl of the resulting supernatant were used per 50 μl PCR reaction

- the remaining supernatant was stored at -20º C and the previous step was repeated when reusing the InstaGene preparation

Only a few PCR bands were obtained after DNA extraction with this method and only for *S. sobrinus*. When using universal streptococci primers, the results were also satisfactory. For both boiling and the InstaGene Matrix methods, pure cultures of *S. mutans* and *S. sobrinus*, Greave's samples and MS agar streptococci suspensions were used.

PureGene DNA Isolation Kit

Whole genomic DNA from *S. mutans* and *S. sobrinus* colonies, respectively, was extracted with the PureGene DNA Isolation Kit. (Gentra Systems, Inc, Minneapolis, USA). This kit produced enough DNA for satisfactory PCR results.

Principle

Bacterial cells are first lyzed with an anionic detergent in the presence of a DNA stabilizer. This functions by limiting the activity of DNases from the cell or environment. RNA is then removed by treatment with an RNA digesting enzyme. Contaminating proteins are removed by salt precipitation. The genomic DNA is finally recovered by precipitation with isopropanol, washed with alcohol and dissolved in a buffered solution containing a DNA stabilizer. The following steps were carried out at room temperature.

Procedure

- the tube with 0.5 ml bacterial suspension was centrifuged at 13,000-16,000 x g for 5 sec to pellet cells

- 300 μl of Cell Suspension Solution were added to the bacterial pellet and the mixture was gently pipetted up and down until the cells were resuspended

- 1.5 μl Lytic Enzyme Solution were added and the tube was inverted 25 times to mix the content

- the sample was incubated at 37ºC for 30 min to digest the cell walls and the tube was inverted occasionally during the incubation

- the sample was centrifuged at 13-16,000 x g for 1 min to pellet the cells and the supernatant was then removed

Cell Lysis

- 300 μl of Cell Lysis Solution were added to the cell pellet and then the mixture was gently pipetted up and down

- the sample was incubated at 80ºC for 5 min to complete the cell lysis and was finally cooled to room temperature

RNase Treatment

- 1.5 μl of RNase solution were added to the cell lysate

- the sample was mixed by inverting the tube 25 times and was incubated at 37º C for 15-60 min

Protein Precipitation

- the sample was cooled to room temperature

- 100μl of Protein Precipitation Solution were added to the RNase treated cell lysate

- the sample was vortexed vigorously at high speed for 20 sec to mix the Protein Precipitation Solution uniformly with the bacterial lysate

- the tube was centrifuged at 13,000-16,000 x g for 3 min and the precipitated proteins formed a tight white pellet

DNA Precipitation

- the supernatant containing the DNA was transferred into a clean 1.5 ml tube containing 300 μl 100% isopropanol

- the sample was mixed by gently inverting the tube 50 times

- the sample was centrifuged at 13,000-16,000 x g for 1 min; the DNA was then visible as a small white pellet

- the supernatant was poured off and the tube drained on clean absorbent paper. 300 μl of 70% ethanol were added and the tube was inverted several times to wash the DNA pellet

- the sample was centrifuged at 13,000-16,000 x g for 1 min and the ethanol was carefully poured off

- the tube was drained on clean absorbent paper and the sample was allowed to air-dry 15 min.

DNA Hydration

- 100 μl of DNA Hydration Solution were added to the DNA pellet

- the DNA was rehydrated overnight at room temperature or by incubating the sample for 1 hour at 65ºC

- the DNA was stored at 4ºC and then used in the PCR

Additional

-for some samples that had few bacterial cells, the DNA concentration obtained was too low for PCR analysis. To overcome this, 0.5μl Glycogen Solution (20 mg/ ml per 300μl 100% isopropanol) were added to maximize the DNA yield

To check whether the DNA concentration was optimal before continuing with the PCR, the DNA was quantified using a spectrofluorimeter (GeneQuant, Pharmacia Biotech).

Isolation of DNA from the Greave's samples

- 1.5 ml of Greave's sample was centrifuged (13,000 x g for 5 sec) and the supernatant was removed with a pipette

- to wash away the Greave's medium, 100 μl TE buffer (Tris buffer 0.01M, Amresco, USA; EDTA 0.001M, pH=8) were used to suspend the pellet

- the suspension was again centrifuged and the procedure was repeated one more time

- the resulting pellet was re-suspended in 0.5 ml TE buffer in a 1.5 ml tube and was used in the PureGene kit procedure (see above)

DNA extraction from Greave's samples was used with satisfactory results for 50 out of 63 of the samples in this study (21 out of 34 positive samples; see page 55). The following two procedures were used during testing and optimizing the method, or in cases with negative or uncertain results (MS agar suspensions use showed 13 more positive samples).

Isolation of DNA from MS agar streptococci suspension

The samples (10μl) were plated on MS agar selective medium. The colonies were

collected and stored in TE buffer at -20C until they were used for the PCR (we used 0.5 ml of the suspension). The steps described above (PureGene kit) were followed.

Isolation of DNA from *S. mutans* and *S. sobrinus* cultures

Overnight bacterial pure cultures (about 0.5-1 billion bacterial cells) were collected and suspended in 0.5 ml TE buffer and the steps mentioned above were followed. This was used as a control in the beginning of the study, while optimizing the method.

Fig. 18 summarizes the DNA extraction methods and the DNA sources used for simple PCR with species-specific primers (see page 39) or nested PCR (see page 41). All the 63 (21x3) PCR samples were DNA extracted from Greave's samples. Forty-two of those showed uncertain or negative results when performing simple PCR. Therefore they were subjected to re-testing by simple PCR or nested PCR using DNA extracted from suspensions of the bacteria harvested from MS agar plates or nested PCR with DNA from the Greave's samples.
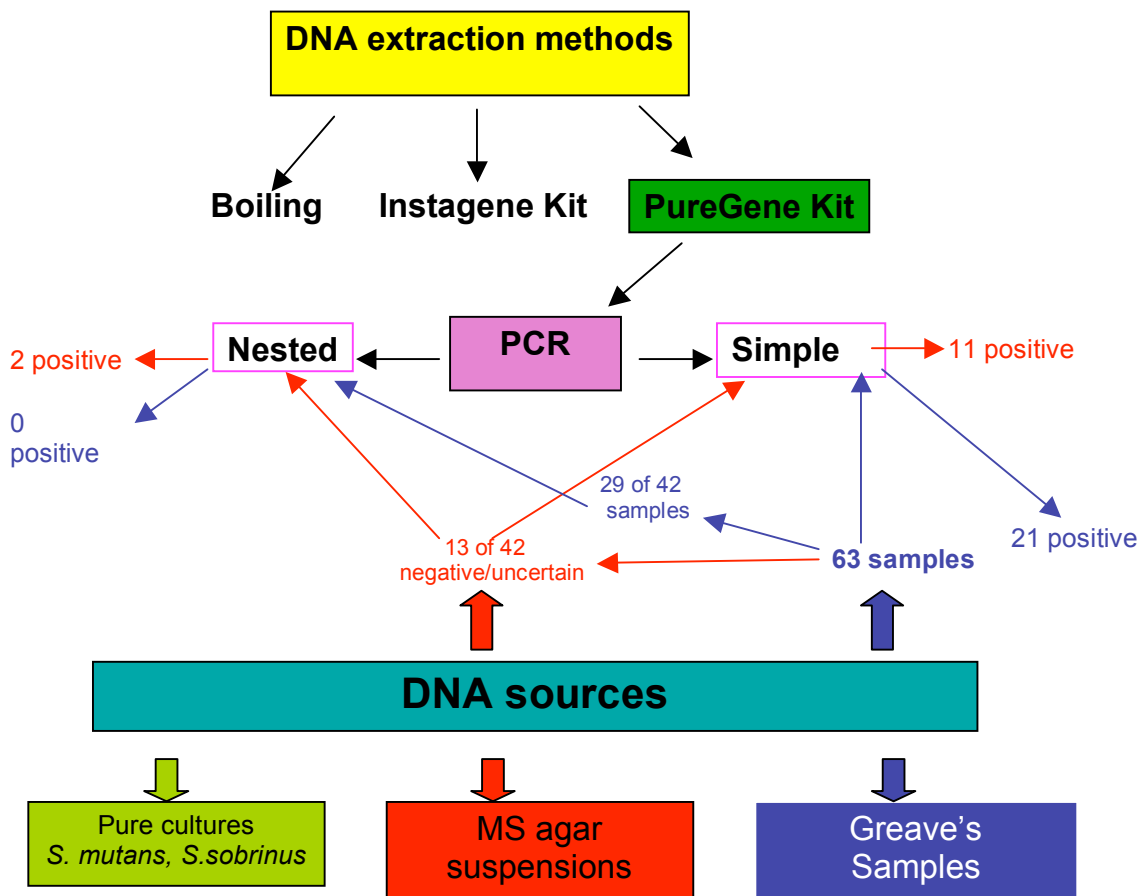
Fig. 18. Sources and extraction methods for bacterial DNA used (bold arrows) in PCR analysis of 63 dentin samples.

PCR primers

The following primers were used:

1. Universal primers for streptococci (degenerate primers) (Poyart et al., 1998) to amplify a 480-bp fragment (fig.31):

d1: 5'-CCI TAY ICI TAY GAY GCI YTI GAR CC-3'

d2: 5'-ARR TAR TAI GCR TGY TCC CAI ACR TC-3'

2. Species-specific primers (simple PCR) for:

- *S. mutans* (Igarashi et al., 1996) to amplify a 1272-bp fragment (fig.29):

SD1. 5'- TAT GCT GCT ATT GGA GGT TC-3'

SD2. 5'- AAG GTT GAG CAA TTG AAT CG-3'

- *S. sobrinus* (Igarashi et al., 1996)) to amplify a 1610-bp fragment (Fig. 29):

SOF 14: 5'- TGC TAT CTT TCC CTA GCA TG-3'

SOR1623. 5'- GGT ATT CGG TTT GAC TGC-3'

3. Species-specific primers (used in nested PCR) for *S. mutans* that were designed using a special software (Lasergene®, Primerselect, DNAstar Inc, USA):

dex A5: 5'- CTT TCC GCG TTA TGC TGC TAT TGG-3'

dex A6: 5'- TTG CTG TTG GCG AGT TTG CTG AT-3'

All the primers were synthesised on request by MedProbe, Oslo.

The species-specific primers were addressed to a portion of the dextranase A gene (*dexA*).

PCR mixtures and cycles

When using the universal primers for streptococci, a fragment of 480-bp length was obtained using DNA extracted with Instagene Kit or the boiling method (Fig. 31). This was used only to test the presence of the streptococcal DNA from the samples.

PCR mixture (25μl):

12.5 μl Hot StarTaq Master Mix

0.25 μl primer D1

0.25 μl primer D2

11 μl distilled water

1 μl DNA

PCR program:

95° C for 15 min

95° C for 30 sec (denaturation)

37° C for 30 sec (annealing) } 40 cycles of amplification

72° C for 90 sec (elongation)

72° C for 10 min

4° C storage

Running time: 1h and 15 min.

Optimization of the species specific PCR method

Preliminary tests included increasing the $MgCl_2$ concentration and different annealing temperatures. To simplify and standardize the procedure and being able to work at room temperature, HotStarTaq[®] Master Mix (Qiagen) was used which is a ready-to-use mixture of Hot DNA Polymerase, Qiagen PCR Buffer and dNTPs. Briefly, 25 μl HotStarTaq Master Mix were pipetted in a PCR tube and then, primers, DNA and water were added to 50 μl. In this study, half of the quantities recommended by the manufacturer were used with good results. Consequently, a 25 μl PCR mixture was used in the PCR machine. In this way, I could analyze more samples with a smaller quantity of HotStarTaq Master Mix.

The HotStarTaq Polymerase from the mixture as supplied is inactive at room temperature. It becomes active by 15 min incubation at 95° C that is incorporated in the PCR program. This prevents formation of misprimed products. Qiagen PCR buffer facilitates the amplification of specific PCR products. It has a very well balanced combination of KCl and $(NH_4)_2SO_4$ providing stringent primer-annealing conditions over

a wider range of annealing temperatures and concentrations than conventional PCR buffers. In the end the following **PCR mixture** (25 μl) was used: 12.5 μl MasterMix, 1.25 μl Forward primer, 1.25 μl Reverse primer, 1.5 μl DNA from samples, and 8.5 μl distilled H_2O.

After optimizing the method and using the HotStarTaq the *final PCR program* was:

95º C for 15 min

94º C for 40 sec

53º C for 1 min   } 40 cycles

72º C for 40 sec

72º C for 15 min

Because the above mentioned program did not show any bands for *S. mutans* when using the boiling method or the InstaGene Kit, before we started using the PureGene Kit, or in case of uncertain results, we tried different other possibilities described below.

Re-PCR for *S. mutans*

In the **first run** DNA from the samples sample and pure cultures was used as a template, together with the primers Dex A5 and SD2, and the HotStarTaq MasterMix. The PCR volume was 25 μl, and the program was 95º C for 15   min; 94º C for 40 sec, 53º C for 1 min, 72º C for 40 sec repeated in 40 cycles; 72º C for 15 min.

The **second run**: the PCR product from the first run (2.5 μl) was used as a template, together with DexA5 and SD2 primers again. PCR volume was 25 μl, and the program was the last one described above, only the annealing temperature was 45º C.

This method amplifies a 1282-bp length fragment.

The nested PCR gave clearer bands though and that is why it was used further to test some uncertain cases.

Nested PCR for *S. mutans* (Fig.30)

First run: DNA from sample/ pure *S. mutans* cultures were used as a template, together with primers: Dex A5 and SD2, and the HotStarTaq MasterMix. PCR volume was 25 μl, and the program was the last one described above.

2nd run: 2.5 μl of the 1st run were used as a template, together with SD1 and DexA6 primers (PCR volume was 25 μl, and the program was the last one described above, with the exception of the annealing temperature that was 45º C initially and then gradually raised to 50º C to make the annealing more specific).

This method gives a 686-bp length fragment.

Firstly the PCR with species-specific primers was tried with DNA from samples. The samples that were uncertain or negative (42 cases), were tested again using the MS agar suspensions from the samples that showed growth on this medium (the DNA quantity is higher when the samples are plated). The samples that were uncertain after this (two cases) were re-tested with nested PCR for *S. mutans* (Fig. 18); our experience was that this strain was more difficult to identify than *S. sobrinus*. Nested PCR was also used to re-test all samples that were negative (after simple PCR) using DNA extracted directly from the Greave's samples (Fig. 18).

Electrophoresis on agar gel

1% agar gel (1 g agar powder dissolved in 100 ml 1xTAE buffer) was used for the electrophoresis. To dissolve the powder, the glass recipient was put in the microwave oven (700W, 1-2 min). Then 1.5 μl ethidium bromide solution (EtBr) was added. The gel was left to harden for at least 30 min. Afterwards the gel tray was transferred to the running chamber and filled with running buffer (1xTAE buffer). The gel had to be submerged under 1mm of solution. 5 μl of the PCR product was then mixed with 1 μl of loading dye and this mixture was applied in the gel wells. The DNA marker (pGem$^{®}$ DNA Markers, Promega, USA) was loaded in one of the wells flanking the sample wells. The power supply was of 100V for 30 min. The results were read in UV light (visualisation of the DNA bands).

Figs. 19 and 20 show the running of the gel.
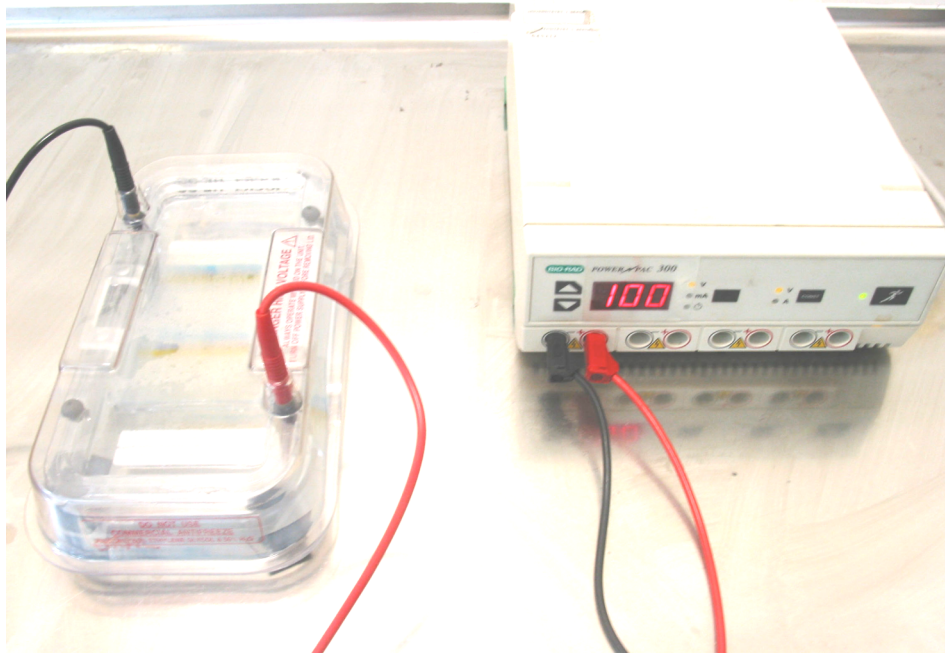
Fig. 19. Running the gel electrophoresis.
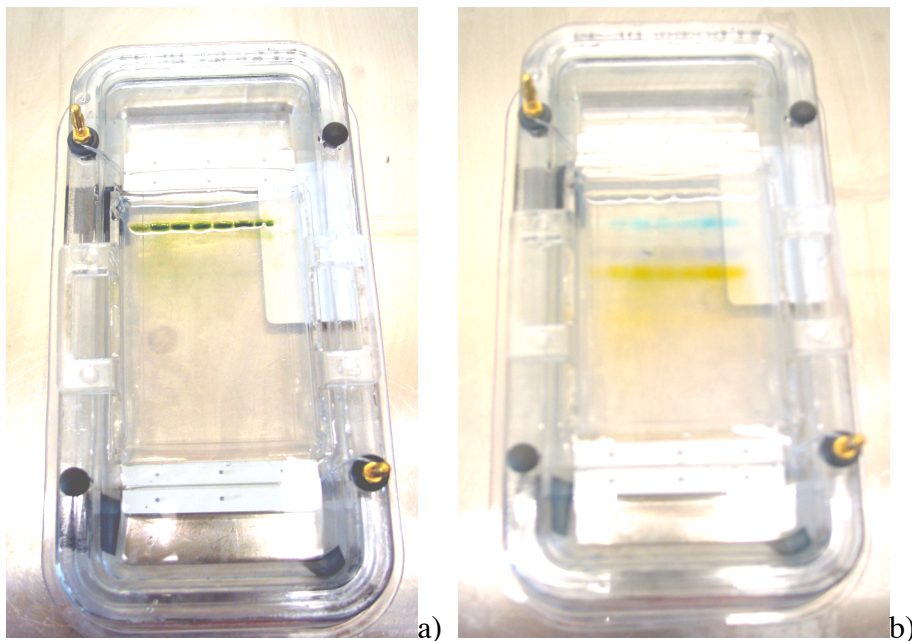
Fig. 20. a) Preparation of gel for electrophoresis; b) the gel after running 30 min

The resulting bands were viewed in UV light and photographed. Using a DNA standard (Fig. 21), we could estimate the length of the resulting fragments.
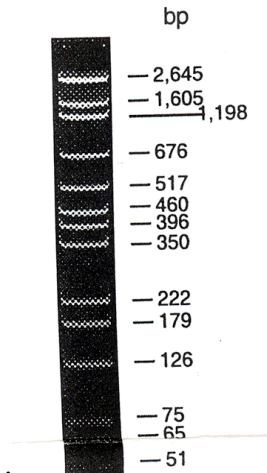
Fig. 21. Standards - corresponding DNA bands for different fragment lengths.

Testing the antimicrobial effect of Carisolv®-2 gel

Bacterial suspensions (100 μl) containing dilutions 1/10 and 1/100 of McFarland standard 0.5, of *S. mutans*, *S. sobrinus*, *L. acidophilus* and *A. naeslundi* were prepared. The two dilutions of *S. mutans, S. sobrinus*, *A. naeslundi* and of *L. acidophilus* were inoculated in triplicate onto nutrient agar (Kneist and Heinrich-Weltzien, 2001), Rogosa agar and supplemented Columbia agar plates with 5% horse serum and 10 ml/litre hemin/menadion solution (500mg hemin, 20 mg menadion / litre). After 1 hour at 4º C, 10 mm reservoirs were cut and the gel removed with a sterile needle. 0.1 ml of Carisolv-2 gel was then applied to each well. The plates were again left 1 hour at 4º C and the gel was allowed to diffuse into the agar. After 24 hours of anaerobic incubation at 37º C, the inhibition zones were measured to the nearest mm.

Statistical analysis

The Wilcoxon Signed Ranks test (for two related samples) was used for analysis of significant weight differences between carious and healthy dentin. The range, 50th percentile and the median of cfu/mg were calculated for each growth medium. For the descriptive purpose, median cfu/mg was used to present the data while for statistical

comparison the mean cfu/mg was more convenient. Percents of the samples that presented different bacterial species were also calculated from the total number of samples, after performing biochemical tests and PCR. For each percent, a confidence interval was calculated (Daniel, 1999). The SPSS software (version 11.0, Chicago, Illinois) was used to perform the analysis. The threshold of significance was chosen for $p \leq 0.05$.

RESULTS

Twenty-one of the 24 teeth showed bacterial growth while three were culture-negative. The DIAGNOdent readings showed numbers between 38-96 for carious dentin (samples 1) and between 4-12 for healthy dentin (samples 2 and 3). For samples with readings below 8, neither bacterial growth, nor PCR bands were obtained (six samples 2 and six samples 3 showed nor bands for streptococci, neither growth for lactobacilli or *Actinomyces*).

Cultivation

Total and selective cfu counts/mg carious dentin and non-carious dentin are shown in Table 1.

GROWTH MEDIA	SAMPLES 1	SAMPLES 2	SAMPLES 3
Blood agar aerobic	3.46×10^4	1.3×10^3	1.1×10^3
	$7.3 \times 10^3 - 1 \times 10^5$	$0 - 4.2 \times 10^3$	$0 - 4.4 \times 10^3$
Blood agar anaerobic	6.93×10^4	2.5×10^3	2.1×10^3
	$3.6 \times 10^4 - 9.6 \times 10^4$	$0 - 4.5 \times 10^3$	$0 - 4.2 \times 10^3$
MS agar	1.98×10^4	0	0
	$0 - 2.8 \times 10^4$	$0 - 3.2 \times 10^3$	$0 - 2.7 \times 10^3$
Rogosa agar	2.5×10^4	1.35×10^3	0
	$0 - 2.9 \times 10^4$	$0 - 4.2 \times 10^3$	$0 - 4.3 \times 10^3$
CFAT agar	8×10^3	0	0
	$0 - 1.8 \times 10^4$	$0 - 2.4 \times 10^3$	$0 - 1 \times 10^3$

Table 1. Median cfu/mg dentin on the various culture media. The data represent the median and range (cfu/mg dentin) of the three types of samples (each individual sample was plated in triplicate and its mean used).

Significant ($p < 0.001$) differences were found between the total aerobic counts for samples 1 (before treatment), samples 2 (after drilling) and samples 3 (after Carisolv-2).

Samples 1 showed the highest bacterial levels while samples 3 had significantly (p<0.05) less bacteria than samples 2. Fig. 22 demonstrates the range, 50th percentile and median values for the total aerobic growth of samples 1, 2 and 3.
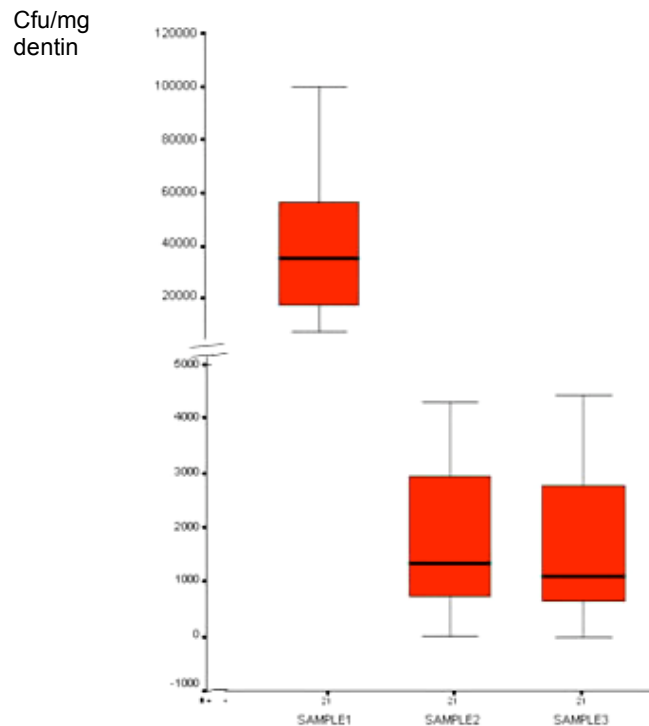
Fig. 22. Box-and-whisker plots comparing aerobic growth on blood agar for samples 1, 2 and 3.

A significant difference was also found when comparing the total anaerobic growth of samples 1, 2 and 3 (p < 0.001) as well as of samples 2 and samples 3 (p = 0.001). Their range, 50th percentile and median growth are shown in Fig. 23.

Cfu/mg dentin
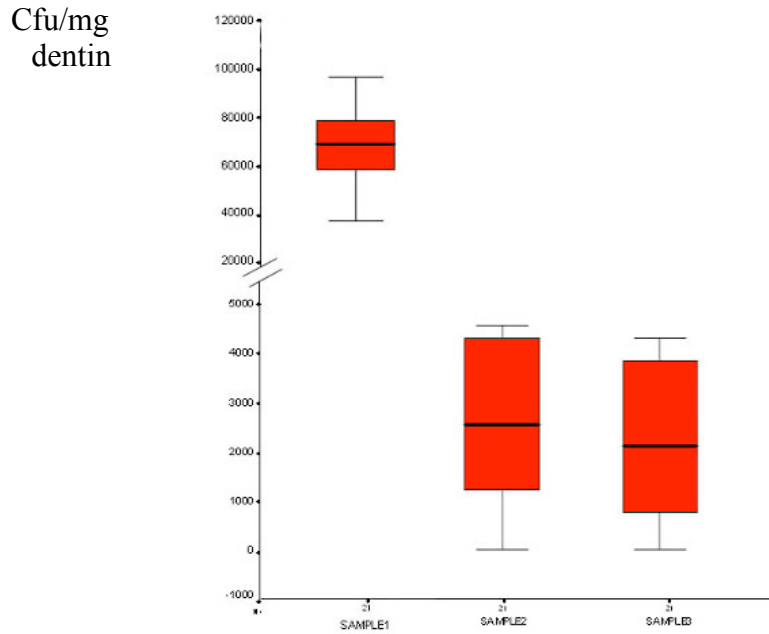
Fig. 23. Box-and-whisker plots comparing anaerobic growth on blood agar for samples 1, 2 and 3.

As shown in Fig. 24, the anaerobic cfu/mg reduction from samples 2 to samples 3 (mean difference ± SE: 326.19±89.8) (b) was bigger than the aerobic one for the two samples (mean difference ± SE: 102.38±43.9) (a) ($p = 0.05$).
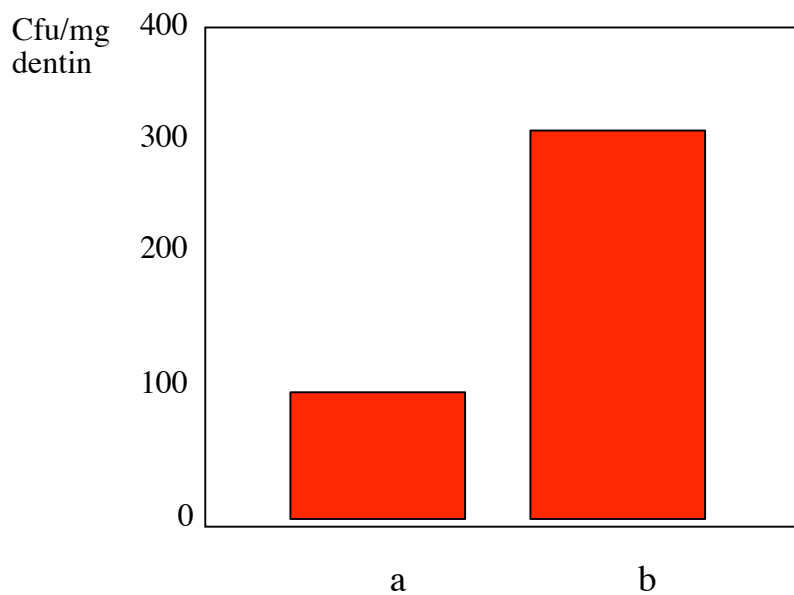
Fig. 24. Mean differences in a) aerobic and b) anaerobic total bacterial counts (cfu/mg dentin) between samples 2 and samples 3.

Comparison of the growth on MS agar revealed a significant difference between samples 1 and samples 2 and between samples1 and 3 (p < 0.001) and no statistical significant difference between samples 2 (mean of 3.8 x 10^3 cfu/mg) and samples 3 (mean of 3.1 x 10^3 cfu/mg). Fig. 25 shows the range, 50th percentile and median growth for samples 1, 2 and 3 on MS agar.
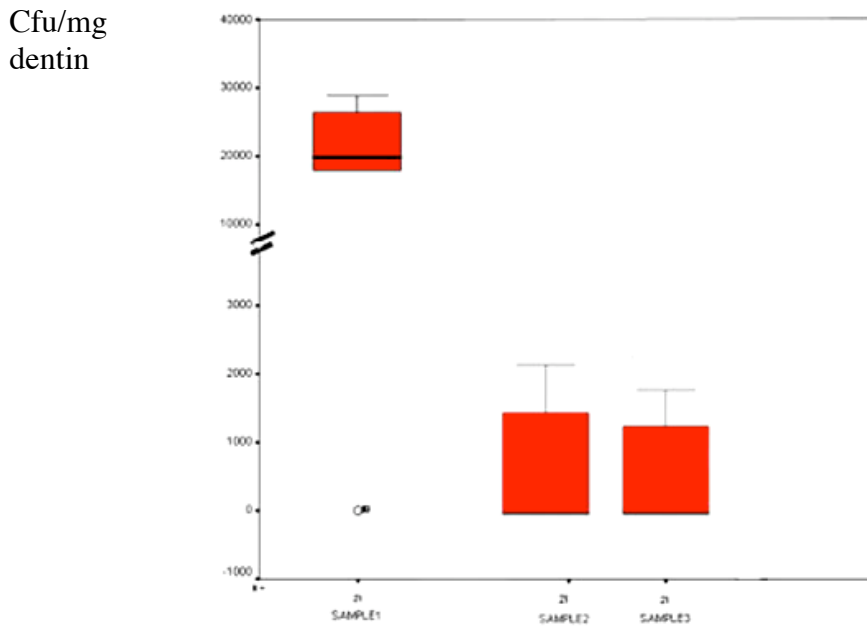
Cfu/mg
dentin

Fig. 25. Box-and-whisker plots comparing MS agar growth for samples 1, 2 and 3.

Highly significant differences were found when comparing cfu/mg counts on Rogosa agar for samples 1 and samples 2 and samples 1 and samples 3 (p<0.001), but weak significance for samples 2 versus samples 3 (p=0.05). Fig 26 shows the range, 50th percentile and median growth of samples 1, 2 and 3 on Rogosa agar.
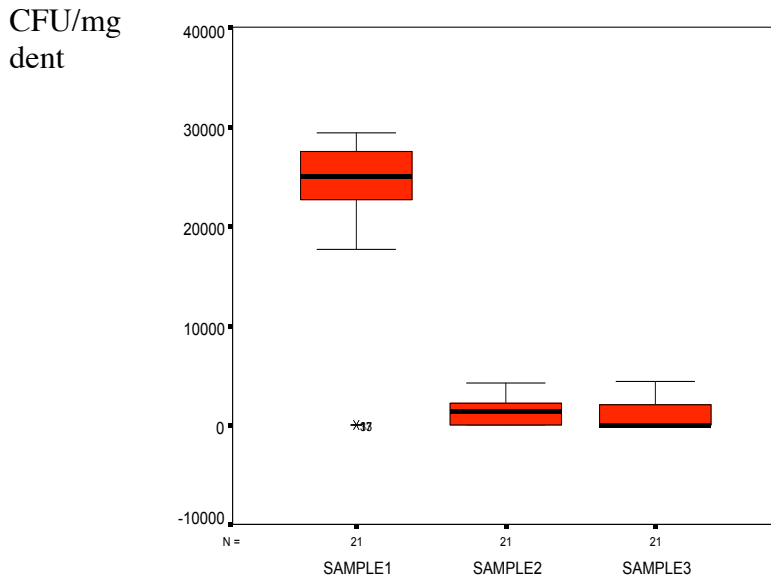
CFU/mg
dent

Fig. 26. Box-and-whisker plots comparing Rogosa agar growth (cfu/mg dentin) for samples 1, 2 and 3.

Significant differences were found when the CFAT growth was compared, samples 1 versus samples 2 and samples 1 versus samples 3 (p = 0.003) and samples 2 versus samples 3 (p < 0.05).

Fig. 27 shows the different ranges, 50th percentiles and medians of growth on CFAT medium.
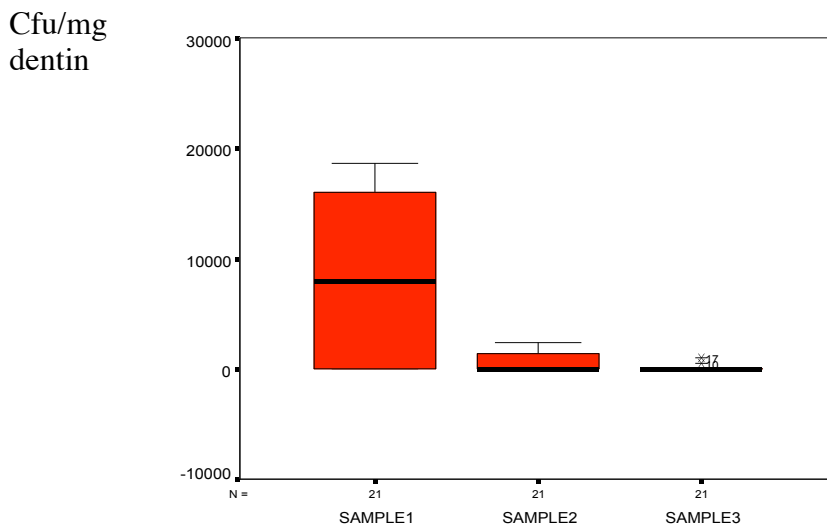
Cfu/mg
dentin

Fig. 27. Box-and-whisker plots comparing CFAT agar growth for samples 1, 2 and 3.

The mean cfu/mg difference between samples 2-samples 3 was also higher on CFAT than on Rogosa agar (p = 0.044)

Fig. 28 illustrates the difference in cfu/mg between samples 2 and samples 3 as revealed on the CFAT (mean difference ± SE: 561.9±172.8) and Rogosa (mean difference ± SE: 85.7± 65.7) media.
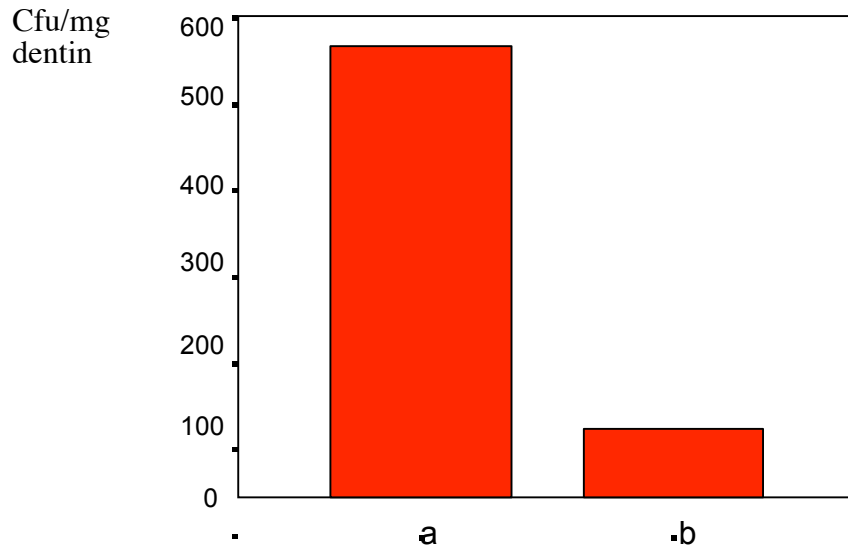
Fig. 28. Mean differences (cfu/mg dentin) between samples 2 and samples 3 assessed on a) CFAT agar and b) Rogosa agar.

Table 2 summarizes the p-values for the cfu/mg comparisons between the samples.

Growth media	Samples 1 versus Samples 2	Samples 1 versus Samples 3	Samples 2 versus Samples 3
Blood agar aerob	.000	.000	.045
Blood agar anaerob	.000	.000	.001
MS agar	.000	.000	.100
Rogosa agar	.000	.000	.050
CFAT	.003	.003	.012

Table 2. Significance levels for cfu/mg differences between the samples on the growth media.

Table 3 shows the number of samples with streptococci, lactobacilli and *Actinomyces* as demonstrated on the selective media used. Before treatment streptococci were found in 76.1% (0.76 ± 0.18), lactobacilli in 85.7% (0.85 ± 0.14) and *Actinomyces* in 52.3% (0.52 ± 0.21) of the teeth. After treatment with drill, streptococci were present in 42.8% (0.42 ± 0.21), lactobacilli in 52.3% (0.52 ± 0.21) and *Actinomyces* in 33.3% (0.33 ± 0.20) of the teeth, while after Carisolv-2 treatment the frequencies were 42.8% (0.42 ± 0.21), 47.6% (0.47 ± 0.21) and 9.5% (0.09 ± 0.12) respectively. The samples that presented no growth for a bacterium in samples 1, showed no growth in samples 2 or 3 either for that bacterium.

Bacteria	Samples 1		Samples 2		Samples 3	
	growth	no growth	growth	no growth	growth	no growth
Oral streptococci	16	5	9	12	9	12
Lactobacilli	18	3	11	10	10	11
Actinomyces	11	10	7	14	2	19

Table 3. Number of teeth, out of in total 21 teeth, according to samples with growth or no growth.

Table 4 presents the number of samples that tested positive for both streptococci and lactobacilli, streptococci and *Actinomyces*, lactobacilli and *Actinomyces*, or all three groups of bacteria.

Samples	Streptococci+ lactobacilli	Streptococci+ actinomyces	Lactobacilli+ actinomyces	Streptococci+ lactobacilli + actinomyces
Samples 1	15	10	10	10
Samples 2	4	6	5	4
Samples 3	3	2	1	1

Table 4. Number of samples that contained 2 or all 3 of the tested groups of bacteria.

Biochemical tests

Table 5 shows the results of the biochemical speciation of lactobacilli and *Actinomyces*. Some samples tested positive for both species of the same bacteria or for both bacteria. *L. acidophilus* was found in 85.7% (0.85 ± 0.14), (18 out of 21), *L casei* in 42.8% (0.42 ±

0.21), *A. naeslundi* in 52.3% (0.52 ± 0.21) and *A. viscous* in 14.2% (0.14 ± 0.14) of the samples before treatment. After treatment with drill, *L. acidophilus* was found in 42.8% (0.42 ± 0.21), *L. casei* in 23.8% (0.23 ± 0.18), *A. naeslundi* in 33% (0.33 ± 0.20) and *A. viscosus* in 0% of the samples. After treatment with Carisolv-2, *L. acidophilus* was found in 28.5% (0.28 ± 0.19), *L. casei* in 19% (0.19 ± 0.16), *A. naeslundi* in 9.5% (0.09 ± 0.12) and *A. viscosus* in 0% of the samples.

Samples	*L. acidophilus*	*L. casei*	*A. naeslundi*	*A. viscosus*
Sample 1	18	9	11	3
Sample 2	9	5	7	0
Sample 3	6	4	2	0

Table 5. Number of samples according to *Lactobacillus* and *Actinomyces* species.

The three of the samples 1 that contained *A. viscosus,* also contained *A. naeslundi*.
 L. acidophilus was found in 18 of the samples 1, nine of them presenting also *L. casei*.
L. acidophilus was also present in nine of the samples 2, three of them containing *L.casei* as well. *L. acidophilus* occurred in six of the samples 3 and three of them also contained *L. casei.* The co-existence of *L. acidophilus* and *A. naeslundi* was found in 10 of the samples 1, five of the samples 2 and one of the samples 3.

PCR
PCR was performed on all samples from the 24 teeth. However, the three culture-negative teeth showed no PCR bands while all the 34 samples that showed growth, also showed some PCR bands, either for *S. mutans* or for *S. sobrinus* or for both.
Fig. 29 shows the corresponding DNA bands for *S. sobrinus* and *S. mutans* obtained with species specific primers.
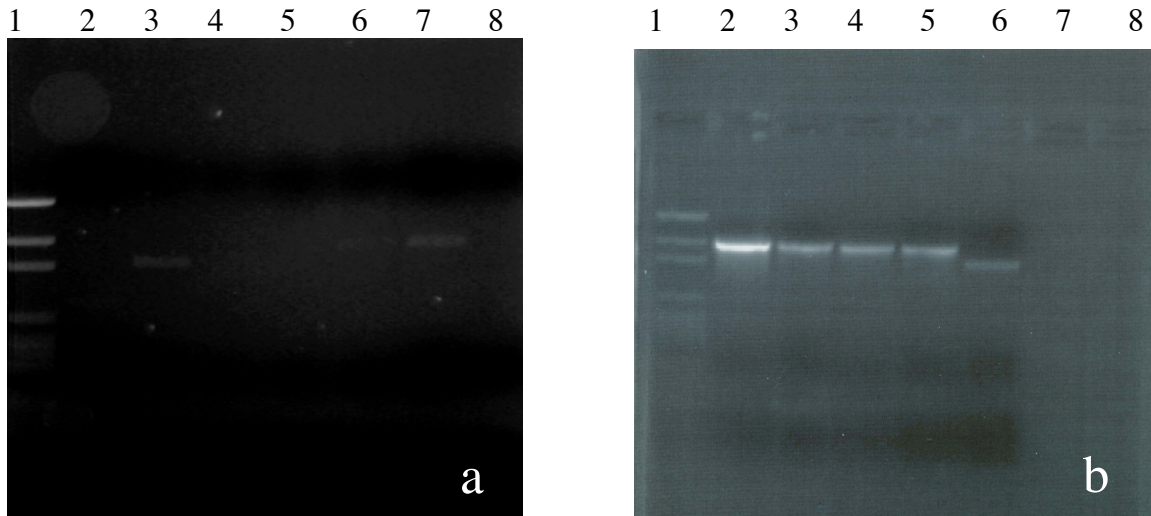
Fig. 29. Simple PCR bands for *S. mutans* (1272-bp fragment length) and *S. sobrinus* (1610-bp fragment length). a) 1- DNA standard; 2- negative sample; 3- positive sample for *S. mutans;* 4, 5- negative samples; 6, 7- positive samples for *S. sobrinus*; 8- negative control. b) 1- DNA standard, 2, 3, 4, 5- *S. sobrinus* positive samples; 6- *S. mutans* positive sample; 7, 8- negative controls.

Fig. 30 shows the band obtained for *S. mutans* when performing nested PCR.
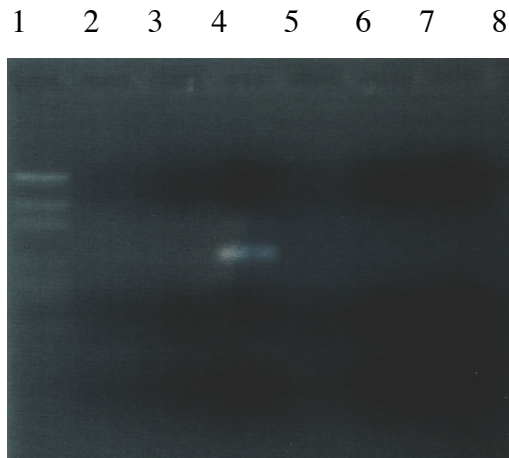
Fig. 30. Nested PCR for *S. mutans* (686-bp fragment length). 1- DNA standard; 2, 3- negative samples; 4- positive sample for *S.mutans*; 5, 6- negative samples; 7, 8- negative controls.

Fig. 31 shows the PCR bands obtained with universal primers for streptococci. This assessed the presence of streptococcal DNA in the samples (DNA obtained only by boiling or Instagene Kit).
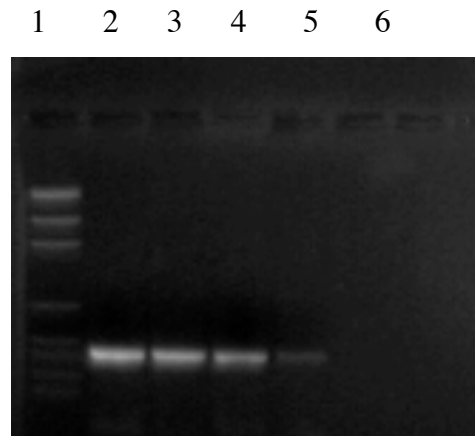
Fig. 31. Fragments (480-bp length) (PCR bands) obtained with degenerate primers. 1- DNA standard; 2, 3, 4, 5- positive samples for streptococcus; 6- negative control.

Table 6 shows the number of samples that tested positive in PCR for *S. mutans, S. sobrinus* or both.

SAMPLES	*S. mutans*	*S. sobrinus*	*S. mutans* and *S. sobrinus*	Total positive samples
Samples 1	2	1	13	16
Samples 2	3	1	5	9
Samples 3	3	2	4	9
Total	8	4	22	34

Table 6. Number of samples that tested positive for *S mutans*, *S sobrinus* or for both.

Before treatment, *S. mutans* was detected in 71.4% (0.71 ± 0.19) and *S. sobrinus* in 66.6% (0.66 ± 0.20) of the samples. After treatment with drill. the corresponding frequencies were 38% (0.38 ± 0.20) and   28.5% (0.28 ± 0.19). Carisolv-2 treatment resulted in 33.3% (0.33 ± 0.20) samples with *S. mutans* and 28.5% (0.28 ± 0.19) with *S sobrinus*. The same sample did not always exhibit both *S. mutans* and *S. sobrinus*. Some samples tested positive for *S. mutans* or *S. sobrinus* alone (Table 6).

All samples were tested using DNA extracted from the Greave's media with simple PCR. Twenty-one samples showed positive results (20 for *S. mutans* and 19 for *S. sobrinus*). The samples that were negative or showed uncertain results were re-tested using suspensions prepared in TE buffer of bacteria harvested from MS agar. This gave 13 more positive samples, 11 for *S. mutans* and six for *S. sobrinus*. In two out of the 11 *S. mutans* identifications, nested PCR was necessary to confirm the uncertain results obtained with simple PCR. Although nested PCR was used to check all the remaining negative samples (29 Greave's samples), it did not show more positive results.

For *S. mutans* and *S. sobrinus* harvested from MS agar, the detection limit of the species specific PCR was 65 cfu/0.1 ml and 54 cfu/0.1 ml respectively.

Fig. 32 shows the total number of PCR bands obtained with DNA from different sources and the number of bands obtained from each samples set (samples 1, 2 and 3).

19 bands *S. mutans.*
>39 bands-21 positive samples - Greave's samples DNA
20 bands *S. sobrinus*

11 bands *S. mutans*
>17 bands–13 positive samples-MS agar suspensions DNA
6 bands *S. sobrinus*

34 samples

56 bands

16 **samples 1**
2 bands– *S. mutans*
1 band– *S. sobrinus*
13 bands-both (13x2)

9 **samples 2**
3 bands- *S. mutans*
1 band- *S. sobrinus*
5 bands-both (5x2)

9 **samples 3**
3 bands- *S. mutans*
2 bands- *S. sobrinus*
4 bands-both (4x2)

Fig. 32. Number of PCR bands according to DNA source and samples group.

Antibacterial effect of Carisolv-2

All *S. mutans* inoculated plates (with two different dilutions) showed growth after 24 hours on nutrient agar and inhibition areas with diameters between 15.7-15.9 mm. None of the *A. naeslundi* inoculated plates showed growth in this agar medium, even after 48 or 72 hours incubation, that is why we decided to use a supplemented Columbia agar. On this medium, *S. mutans* showed inhibition areas between 16.5-18 mm and *S. sobrinus* 15-16 mm. The inhibition areas for *A. naeslundi* were between 32-35 mm (Fig. 34c) and for *L. acidophilus* between 17-18 mm. The smaller the bacterial concentration was, the larger inhibition areas were formed.

For *L. acidophilus*, on the Rogosa plates the inhibition areas presented a diameter between 16.5-17 mm for the two tested bacterial concentrations (Fig. 34a and 34b).

Table 7 shows the inhibition zones for the cariogenic bacteria grown on supplemented Columbia agar, Rogosa agar and simple nutrient agar.

Growth medium	*S. mutans*	*S. sobrinus*	*A. naeslundi*	*L. acidophilus*
Supplemented Columbia agar	17±0.3	15.8±0.2	34±1.2	17.6±0.1
Rogosa agar	-	-	-	16.8±0.2
Nutrient agar	15.8±0.2	-	-	-

Table 7. Inhibition area diameters (mm) for the cariogenic bacteria on different culture media. Data show the mean ± SE of three parallels, when the bacterial concentration was 5×10^4 cfu/ml.

Fig. 33 illustrates the inhibition area obtained by applying the Carisolv gel in the reservoirs on the inoculated Rogosa agar and supplemented Columbia agar.

a)

b)

Fig. 33. Inhibition area around the reservoirs containing the Carisolv gel: a) on Rogosa agar; b) on supplemented Columbia agar for *A. naeslundi*.

Samples weight

The mean weight of carious dentin was 0.272 ± 0.03 mg and of non-carious dentin 0.370 ± 0.05 mg. The difference was significant (p <0.001, Wilcoxon test). The amount of dentin in 1 ml was 0.151 mg/ml for carious dentin and 0.205 mg/ml for healthy dentin.

DISCUSSION

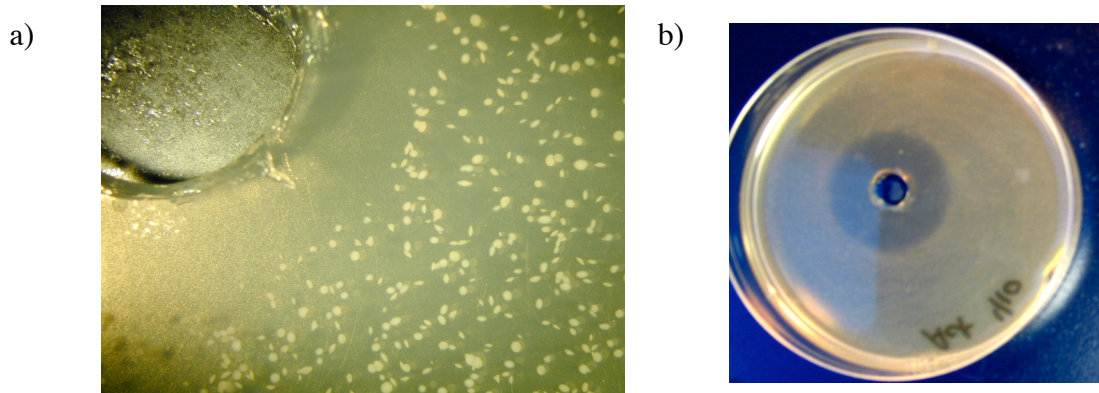The aim of this *in vitro* study was primarily to investigate the antimicrobial effect of the Carisolv-2 gel because such information is lacking in the literature. It was done by comparison of bacterial levels in dentin samples from extracted teeth with carious lesions, taken before and after application of the gel, and by assessing the susceptibility of principal cariogenic bacteria (*S. mutans, S. sobrinus, L. acidophilus, L. casei,* and *A. naeslundi)* to the gel. Since drilling is the standard invasive treatment method, the bacterial levels of adjacent non-carious dentin, obtained after all carious dentin had been removed by drilling or the Carisolv-2 method, were also compared.

The results showed that from a microbiologically point of view, caries treatment by the Carisolv-2 system was more efficient than drilling. Also, the Carisolv-2 gel had antibacterial effect. These findings do not support the null hypothesis and are therefore in line with my work hypothesis.

Antibacterial effect

Carious dentin showed significantly higher cfu/mg counts and more samples contained *S. mutans* and *S. sobrinus* than non-carious dentin did. Similar findings have been reported (Bjørndal and Larsen, 2000; Baysan et al., 2000; Lager et al., 2003; Azrak et al., 2004). Non-carious dentin from Carisolv-2 treated lesions demonstrated on an average the significantly lowest such counts. This indicates that the gel has *in situ* antibacterial effect. This is in accordance with results from Carisolv-1 studies in adults (Lager et al., 2003) and in children (Azrak et al., 2004). Since the latter gel was not included in my study, a direct comparison of the antimicrobial efficacy of the two gels could not be done. Comparison of results from different studies would require a systematic review including strict criteria for acceptance of comparable results. Interestingly, the *in situ* reduction in bacterial counts of Carisolv-2 treated dentin was more pronounced on anaerobic than on aerobic blood agar. Also, lactobacilli and *Actinomyces* showed a significant cfu/mg reduction when comparing samples 2 and 3, while cariogenic streptococci did not. Nor was there a significant difference between samples 2 and samples 3 concerning frequency

of *S. mutans* and *S. sobrinus*. This indicates that the Carisolv-2 gel inhibits more the growth of anaerobic than aerobic bacteria. To verify this hypothesis a study including more anaerobic and aerobic species is in progress. The results we have obtained until now, however tend to confirm this hypothesis. The inhibition areas obtained for *L. acidophilus* and especially for *A. naeslundi* were larger than those for streptococci on the supplemented Columbia agar.

Kneist and Heinrich-Weltzien (2001) inoculated nutrient agar with over-night cultures of *S. mutans, S. sobrinus, L. casei* and *A. naeslundi* for testing the antimicrobial effect of Carisol-1 gel. They found that the gel produced inhibition zones of 13-15 mm which is the lower limit of the range for our results (see Table 7).

The clinical importance of infected dentin remaining after carious treatment is still not well known. It has been reported, however, that about 10^2 cfu/ml of remaining bacteria in dentin from the cavity floor is considered clinically insignificant (Bjørndal et al.,1997; Kidd et al.,1993). In our study, there was on an average 0.20 mg non-carious dentin/m1 Greave's medium which corresponds to approximately 5×10^2 bacteria cfu/ml. It is not realistic to indicate whether this bacterial level predisposes *per se* to secondary caries. More important is the quality of the filling covering the infected dentin. What is clear is that leaking fillings represent a caries risk.

Biochemical tests

The biochemical tests were used to speciate *Actinomyces* recovered from CFAT plates. Three samples tested positive for both *A. viscosus*, and *A. naeslundi* (see Table 5). It could have been, however, the same species because it is not easy to distinguish them biochemically. Furthermore, *A. viscosus* has been considered a subgroup of *A. naeslundi* (Marsh and Martin, 1999).

I was not able to identify *S. mutans* and *S, sobrinus* using the biochemical test kit. I therefore decided to try PCR identification.

PCR

Before PCR methods were available, detection of *S. mutans* and *S. sobrinus* had to be done by conventional culture methods plus biochemical reactions (for example,

production of acids from N-acetylglucosamine, the presence of -galactosidase and -glucosidase and/or serological analyses). These methods are time-consuming and sometimes unstable. That is why genetic approaches are recommended (Sato et al., 2003) and I decided to use PCR diagnostics.

PCR with species-specific primers (Igarashi et al., 1996) and nested PCR of two samples in which simple PCR was not successful, identified *S. mutans* and *S. sobrinus*. Quantification of the species would have been possible if real-time (RT)-PCR had been available.

PCR has the big advantage over culture techniques that the bacteria do not need to be alive and cultivable, and the samples can remain stable over long periods of freezing. Recent studies comparing RT-PCR techniques with traditional culture methods have shown that the former techniques detect up to 40 times more anaerobic bacteria than traditional cultivation (Martin et al., 2002; Nadkarni et al., 2002). If the DNA quantity in the samples is too low, mainly if inappropriate DNA extraction methods are used, RT-PCR could result in underestimation of the number of bacteria (Yano et al, 2002).

Species specific PCR based on dextranase (Igarashi et al., 2000) or glucosyltransferase (Yano et al., 2002) have been proved to be very effective methods for detection of these strains. However the disadvantage of these direct PCR methods is the potential lower sensitivity for directly detecting bacteria from clinical samples; this was experienced in previous studies (Sato et al., 2003) and we met the same problem for a few samples. For these samples, with a low amount of bacteria (DNA) the nested PCR we established has proved to be more sensitive. Using a nested PCR, we obtained bands even when using only the Instagene® kit, still the bands were clearer when using the PureGene® kit.

Weight and volume of dentin

This study results showed that healthy dentin is heavier than carious dentin. This is in accordance with previous findings (Lager et al, 2003). The explanation could be that healthy dentin has more minerals and therefore weighs more, even if the volumes are the same. However, other studies found the opposite, namely a slight tendency of quantitatively more demineralized dentin than sound (hard) dentin in samples having the same weight (Bjørndal et al., 1997). Weerheijm and co-workers (1999) reported

approximate equal weights of carious and healthy dentin using a similar method, but this could be due to different sampling procedures and the dentin different drying time (Lager et al., 2003).

Methodological considerations

The study design

Using two halves of the same carious lesion for pair-wise comparison of the treatment effects on the microflora can be considered a strength of my study. This was one reason why I chose this *in vitro* study. A corresponding *in vivo* model would not have been feasible. The median was used to describe the data instead of the mean that would have been more affected by the lack of a normal distribution and skewness of the data. Mean was used only to describe the difference between sample 2 and 3 because there were many values equal to 0. In such cases the median was 0 too and did not offer sufficient information for this comparison.

Selective media

Even though the media used to isolate streptococci, lactobacilli and *Actinomyces* spp. are referred to as selective media, they proved not to be absolutely selective for the groups of bacteria that I was looking for. Consequently, colony morphology and gram-stained smears helped to identify the groups of bacteria.

PCR

One experience from my master's study is that elaborating PCR protocols may be time-consuming and represent steps of "try and fail".

It was not always possible to obtain the expected bands with simple PCR, mainly because of the low DNA quantity in some samples. Nested PCR has proved to be very useful, giving satisfactory results in such cases.

Bacterial counts

Bacterial counts in dentin have been referred to as cfu/ml bacteria suspension (Lager et al, 2003) and cfu/mg dentin (Baysan et al., 2000). By weighing my dentin samples it became clear that their weights varied. Transferring samples of different weights to individual vials with the same volume of Greave's medium and then reporting the number of bacteria as cfu/ml would have been more inaccurate than cfu/mg dentin. For

comparison, I calculated my bacterial counts both as cfu/ml and cfu/mg. Significance testing of sample differences using both units of measure showed, however, the same p-values.

I used DIAGNOdent and dental probes to differentiate carious and healthy dentin. It has recently been shown that dentin samples with DIAGNOdent values of 15.6 contained no bacteria as assessed by PCR using universal primers based on the nucleotide sequence of a conserved region of the 16S rDNA (Iwami et al., 2004). However, this method can not be considered as sensitive as species specific PCR that we performed for *S. mutans* and *S. sobrinus*. For the teeth used in this study, DIAGNOdent showed readings between 38-96 for carious dentin and between 4-12 for healthy dentin; for samples below 8 we could not detect any bacteria. This was the case for six samples 2 and six samples 3. However, we did not perform PCR for other bacteria than *S. mutans* and *S. sobrinus*. This might have revealed for example, the presence of *L. acidophilus* or *A. naeslundi*, in case cultivation was not accurate. Even so, there is still a problem, that PCR does not distinguish between dead bacteria (left in the cavity after treatment) and alive bacteria.

Table 8 summarizes some of the problems that I faced and how I tried to solve them.

Procedure	Pitfall	Solution
Sample collection	Different microflora in samples 2 and samples 3	Each tooth was split vertically in two halves through the middle of the carious lesion
Selection of carious lesions	The microflora differs with the type of caries lesion	Used only class I and II cavities.
Age of the test subjects	Age-related differences between children and adults	Used only patients aged 18-52.
Best preservation medium for the dentin samples	If the medium is inappropriate, bacteria will not survive or their numbers will increases or decreases	Pilot study using four media to find out which one keeps the number of bacteria constant and Greave's

	during the storage	medium was chosen
Identification of *S. mutans* and *S. sobrinus*	Biochemical tests gave confusing results	Established PCR methods for the dentin samples
DNA extraction	Boiling and rapid kits are insufficient for DNA extraction from *S. mutans*	The PureGene Kit gives good results
Simple PCR (using species specific primers)	Unclear or no bands	Nested PCR was successful

Table 8. Problems met and solutions found during my study.

Future perspectives

There is need for more in-depth examination to assess the presence and correlations between more bacteria in carious lesions than the three cariogenic bacteria I studied. Such studies should include cooperation between dentists, bacteriologists and chemists to provide adequate expertise. My results showed that compared to drilling, the Carisolv-2 treatment reduced significantly the bacteria in hard dentin adjacent to the dental lesions and other studies have shown that patients prefer Carisolv treatment rather than drilling. A challenge will therefore be to convince more dentists to use the Carisolv system.

CONCLUSIONS

1. The findings support my work hypothesis that the Carisolv-2 system is a better antibacterial alternative for caries treatment than drilling (in selected cases) and that the Carisolv-2 gel is the antibacterial agent.

2. Carious dentin contained significantly more bacteria than non-carious dentin did.

3. Pair-wise treatment of the carious lesions with conventional drilling or the Carisolv-2 system resulted in significantly lower bacterial levels in specimens of adjacent non-carious dentin from Carisolv-2 treated caries lesions.

4. The Carisolv-2 gel inhibited the growth of the principal cariogenic bacteria (*S. mutans*, *S. sobrinus*, *L. acidophilus*, *A. naeslundi*) and demonstrated inhibition zones larger then those reported for the Carisolv-1 gel.

5. PCR was used successfully to identify *S. mutans* and *S. sobrinus* in the samples.

6. It is hypothesized that the Carisolv-2 gel is more antibacterial against anaerobe than aerobe bacteria, especially against *A. naeslundi*.

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Appendix A

Composition of the preservation fluid media.

Greave's medium

Glutamic acid Na-salt	50 g
Water RO-water	700 ml
Bovine serum albumin	50 g
Water free glycerol	100 ml
RO-water up to	1000 ml

Skim milk

RO-water (ca 30-50C)	1000 ml
Skim milk (Oxoid L-31)	200 g

Sterilization in autoclave at 121° C , maximum 13 min.
The lid has to be unscrewed again after sterilization.

VMG II (Møller 1966)

A) Agar Nobel   0.1 g
   Distilled water
   The agar dissolves in water through warming.

B) Bacto-gelatin   10 g

Bacto-Tryptose	0.5 g
Thiotone	0.5 g
L(+)-cysteine hydrochloride	0.5 g
Thioglycollic acid or	0.5 ml
Natriumthio-glycolate	0.6 g
Bacteriological charcoal	10 g

Components of part B have to be dissolved in part A.

C) Salt stock solution II

Phenylmercuric acetate	0.03 g
$CaCl_2 \cdot 6H_2O$	2.4 g
Kcl	4.2 g
NaCl	10 g
$MgSO_4 \cdot 7H_2O$	1 g
Sodium glycerophosphate	100 g
Glass-distilled water to	1000 ml

Part C must have a pH = 7.5 with 1-N NaOH.
The medium has to be autoclaved for 20 min.

Appendix B
Composition of the cultivation media

`MS agar` is a differential medium containing dyes, nutrients, 5% sucrose and growth inhibitors for organisms other than streptococci.

`CFAT` is used as a selective medium for *Actinomyces*; neutral acriflavin and potassium tellurite are used in combination with the known selective agents cadmium and fluoride to eliminate most of the competing plaque flora.

Composition of CFAT per liter was as follows: Trypticase soy broth (BBL Microbiology Systems), 30 g; glucose, 5 g; agar, 15 g' cadmium sulfate, 13 mg; sodium fluoride, 85 mg; neutral acriflavin, 1.20 mg; potassium tellurite, 2.50 mg; basic fuchsin, 1.25 mg; defibrinated sheep blood, 50 ml (Zylber and Jordan, 1982).

Appendix C
Principle of the biochemical tests

Rapid ID 32 A

Principle: The *rapid ID 32 A* strip consists of 32 cupules, 29 of which are used as test cupules and contain dehydrated test substrates. After adding the bacterial suspension, 4 hours of incubation in aerobic conditions are necessary before reading the results.

Content of the kit: 25 rapid ID 32 A strips; 25 incubation lids, 25 result sheets.

Rapid ID 32 STREP

Principle: the rapid ID 32 STREP consists of 32 test cupules with dehydrated test substrates. After 4 hours of incubation the reactions are read either manually or using special apparatus.

Content of the kit: 26 rapid ID32 STREP strips, 25 incubation lids, 25 result sheets.

Appendix D
PCR principle

PCR uses semiconservative replication of DNA to amplify small amounts of existing DNA. Only if the DNA of the bacterial species of interest is present in the sample will the oligonucleotides be able to prime the synthesis of a DNA fragment of a certain size and thus allow the diagnosis. Previous isolation of a pure culture it is not necessary.

DNA polymerase uses single stranded DNA as a template for the synthesis of a complementary new strand. The single stranded DNA is obtained by heating the double stranded DNA to temperatures near boiling. The starting point for the DNA synthesis is specified by supplying an oligonucleotide primer that anneals to that point at a certain temperature (annealing temperature). This is one of the most important features of PCR, that the DNA polymerase can be directed, using specific primers, to synthesise a specific region of DNA.

The reaction mixture is heated repeatedly (20-40 cycles) to separate the original and newly synthesised strands, which become available for further cycles of primer hybridization, DNA synthesis and strand separation. The result of a PCR is that by the end the reaction it contains many (2^n, where n is the number of cycles) double stranded DNA molecules that are copies of the DNA sequence between the primers. This is another important characteristic of the method, the PCR results in the amplification of a specified region (Watson et al., 1992).

The PCR mixture must contain: the two oligonucleotide primers, the enzyme: DNA polymerase, the PCR buffer and a mixture of all 4 deoxynucleotide precursors (dNTPs). To this mixture the DNA is added (small Eppendorf tubes are used with a reacton volume of 50 or 25 μl). Then the PCR cycle is started.

The DNA polymerase or *taq*-polymerase, is a big improvement in the method. The enzyme belongs to the bacterium *Thermus aquaticus* that lives in hot springs at 75º C. The enzyme has its optimal working temperature at 72º C but it is stable until 94º C. It can be added just once in the beginning of the reaction. This allowed the automation of the PCR cycles. Before *E. coli* DNA polymerase was used, but it was destroyed at the

high temperature needed to separate the double stranded DNA and fresh enzyme had to be added for each cycle manually.

The *taq*-polymerase also improves the specificity and sensitivity of the method. Because of *E. coli* enzyme, low temperature had to be used and the primers could anneal at sites where sequences differed slightly from the target sequences. In contrast, the annealing of primers to a different site is considerably reduced when using higher temperatures with the *Taq*-polymerase.

There are some mismatches in this process, of course. Naturally, the DNA replication machinery removes the incorrect added nucleotides, but in vitro, the *Taq*-polymerase does not have this proofreading capability. However, the wrong incorporated nucleotides are not in a significant number (1 for every 2×10^4 nucleotides incorporated) (Watson et al., 1992).