Insights into the bacterial and fungal ecology of endodontic infections
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BACTERIAL ENDOSPORES WITHIN ROOT CANAL INFECTIONS: A PILOT STUDY

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Spire on the roof of Børsen in Copenhagen, Denmark.

The spire is shaped as the tails of four dragons twined together. They should guard the building against enemy attacks. Similarly, sporulation may preserve bacteria.
ABSTRACT

AIM
Some of the bacteria found in root canal infections are capable of forming spores, which enables them to survive harsh environments. Thus far, no study has identified bacterial spores residing in the root canal system. This pilot study aimed to identify bacterial spores within root canal infections.

METHODOLOGY
Fifteen single rooted teeth with apical periodontitis in 10 adult patients were sampled under aseptic conditions using paper points. Samples were incubated 30 min at 80°C and then cultivated anaerobically and aerobically. Sporulation was examined by microscopy and by cultivation in chopped meat carbohydrate broth. Isolates were further characterized using biochemical tests and identified by 16S rRNA sequencing.

RESULTS
Six samples had to be discarded because of contaminated sterility checks. Of the 10 isolated bacteria, only the 6 remaining bacilli were further studied. None of these were spore-forming. Using sequencing, the isolates were identified as *Propionibacterium acnes* and *Propionibacterium avidum*.

CONCLUSION
Within the limitations of this study, no bacterial spores could be detected within root canal infections. More sensitive techniques should be applied on a larger sample size to definitively dismiss the presence of bacterial spores within root canal infections.
INTRODUCTION
Microbial infection of the root canal system causes an inflammatory reaction in the periapical tissues (Kakehashi et al. 1965). Bacteria are the main contributors to this infection, both in quantity and diversity (Özok et al. 2012). The inflammation will not resolve spontaneously, because the host’s immune system cannot infiltrate the infected root canal system and, consequently, is unable to inactivate the bacterial infection. Therefore, effective root canal treatment aims at inactivation and removal of microorganisms and their toxic by-products. However, current techniques are unable to achieve sterility (Nair et al. 1990) because of the intricate network of the root canal system (Wolf et al. 2016) and the protective biofilm community (Stojicic et al. 2013), which both result in hindrance of the disinfectant’s effectivity (Portenier et al. 2002). This may lead to persistent inflammations in the periapical tissues (Nair et al. 1990).

Recently, the microbiome of root canal infections has been extensively mapped using deep sequencing techniques (Li et al. 2010; Özok et al. 2012; Hong et al. 2013). Some of the identified taxa were Anoxybacillus, Bacillus, Clostridium and Filifactor. These taxa contributed 0.05 - 1.31% to the total DNA yield and were present in 65 - 78% of the root canals (Özok et al. 2012). These genera are Gram-positive bacilli and most species are capable of forming endospores. Previous research has identified bacterial spores within saliva (Hall 1925), which provides an inoculation route towards the root canal system.

Spore-forming is a microbial survival mechanism (Vreeland et al. 2000). Some bacteria form endospores within their cell leaving a latent, reproductive structure (Madigan et al. 1997). The tough and thick spore cell wall enables the endospore to survive harsh conditions, such as acidity, chemical disinfectants, drought, heat and radiation. An endospore can remain dormant for many years, but can convert back into a vegetative state when the environment is suitable (Vreeland et al. 2000).

If bacteria are actually forming endospores within root canals, then they will be extra resilient to root canal disinfection and nutrient-depleted conditions, on top of all other survival mechanisms available to microorganisms. Once the harsh conditions have passed, the spores can return to their vegetative state. Subsequently, they can continue to evoke inflammation in the periapical tissues and lead to treatment-resistant inflammation. Because spores are resilient to current disinfectants (McDonnell & Russell 1999), extra antimicrobial strategies will be required to disinfect the root canal system in case their presence would be confirmed. Only one previous study directly examined samples of root canal infections using microscopy, but was unable to identify spores (Kantz & Henry 1974). Therefore, this study aimed to identify bacterial endospores within infected root canals.
METHODOLOGY

Study participants
The study was approved by the Medical Ethics Review Committee of VU University Medical Center, Amsterdam (2011/077). Ten patients attending the endodontic clinic of the Academic Centre for Dentistry Amsterdam (University of Amsterdam and Vrije Universiteit Amsterdam, Amsterdam, the Netherlands) were invited to participate in this study. Patients had fifteen single rooted permanent teeth in need of a root canal treatment or retreatment. Apical periodontitis was confirmed on a periapical radiograph and with a negative sensibility test using ethyl chloride. Teeth had no advanced periodontal involvement, no pulp exposure and no suspected fracture.

Sample collection and cultivation
Samples were collected according to a protocol, modified from Möller (1966). The tooth was isolated using rubber dam and cleaned with pumice. All subsequent procedures were performed aseptically. The tooth, clamp and rubberdam were disinfected by scrubbing with a gauze soaked in 30% (v/v) hydrogen peroxide (H₂O₂; Merck, Darmstadt, Germany) for 2 min. The solution was inactivated by wiping the area with 5% (w/v) sodium thiosulphate (VWR, Fontenay-sous-Bois, France) for 2 min. After preparation of the access cavity and just before entering the pulp chamber, the decontamination procedures were repeated. Sterility of the tooth surface was checked using a sterile microbrush (Microbrush International, Grafton, WI, USA). The brush was placed in a 2 mL Eppendorf tube without fluids, and the handle was cut off with disinfected scissors. The tube was closed and placed on ice.

After preparation of the endodontic access, the canal was instrumented up to a #20 K-file (Dentsply Maillefer, Ballaigues, Switzerland) to the estimated working length as calculated from the preoperative radiograph. In retreatment cases the root canal filling material was removed mechanically and without any solvents prior to sampling. The instrumented canals were filled with sterilized water (MilliQ, Millipore Corporation, Billerica, MA, USA) and five consecutive sterile #20 paper points (Dentsply Maillefer) were each used for 5 s to retrieve the sample from the canal. All files used during the instrumentation of which the handles were cut off, gutta-percha retrieved during retreatment cases and soaked paper points were collected in 25 mL tubes and place on ice. From here on the root canal treatment was continued as usual.

Samples were immediately transported to the laboratory for processing. First, 1 mL MilliQ was added to the samples, and these were vortexed and sonicated (Ultrasonic cleaner 5510E-MT; Branson Ultrasonics Corporation, Danbury, CT, USA)
for 5 min. These samples were incubated for 30 min at 80°C using a water bath (TW8; Julabo Labortechnik, Saalbach, Germany) (Hall 1925). If bacteria were present as spores, they are resilient enough to survive this treatment, whereas all other bacteria will be killed. Of both the samples and untreated sterility checks 500-µL aliquots were dispersed in duplicate on Tryptic Soy Agar (BD Difco, Becton, Dickinson, Le Pont de Claix, France) supplemented with defibrinated sheep blood (50 mL/L; biotrading Benelux, Mijdrecht, the Netherlands), hemin (5 mg/L; Sigma-Aldrich, St Louis, MO, USA) and menadione (0.5 mg/L; Sigma-Aldrich, Steinheim, Germany). Plates were incubated under either aerobic or anaerobic conditions (80% N₂, 10% H₂ and 10% CO₂) at 37°C for 5 - 7 days.

**Microbial determination**

Each morphologically distinct isolate was collected for further determination. Schaeffer-Fulton stains (Schaeffer & Fulton 1933) and Gram stains were prepared to determine endospores and bacterial cell morphology, respectively. Specimens were examined using light microscopy (Axioskop; Carl Zeiss, Jena, Germany; 1000x magnification). Isolates were grown in chopped meat carbohydrate broth, PR II (BD, Becton, Dickinson, Sparks, MD, USA) at 37°C for 7 days, because this medium evokes sporulation and should aid in detecting spore-forming bacteria. The cultures were then stained again to detect endospores.

Biochemical identification of anaerobic microorganisms was performed using API rapid ID 32A (Bio Merieux, Marcy-l’Etoile, France). All macro- and microscopic morphologically distinct isolates that could not be identified using the aforementioned techniques were commercially sequenced using the 16S rRNA-procedure (MicroSeq®, Applied Systems; BaseClear, Leiden, the Netherlands).

**RESULTS**

A total of 15 samples were analysed, of which 6 had to be excluded because of contaminated sterility checks. The remaining 9 samples came from primary root canal infections. Only 3 samples were positive and yielded 10 isolates; 4 were cocci and 6 were bacilli (Table 2.2).

The Schaeffer-Fulton stain was negative for all isolates. Only the 6 bacilli were further analysed, as cocci are unable to sporulate. All isolates showed significant growth in chopped meat carbohydrate broth, although in none of the cultures bacterial spores were detected using the Schaeffer-Fulton stain. As API tests gave inconclusive results, two morphologically distinct isolates (sp8-3, sp15-1) were sequenced. The isolates were assigned a taxonomical rank to >99% similarity level, and, thus, could be assigned to species level (Table 2.2).
DISCUSSION

We hypothesized that bacteria in root canal infections are capable of sporulation. However, within the limitations of this study, bacterial endospores could not be detected within root canal infections. This result is similar to a previous study, although microscopy instead of cultivation was used to identify spores (Kantz & Henry 1974). In the current study, only \textit{Propionibacterium acnes} and \textit{Propionibacterium avidum} were detected within 9 heat-treated root canal samples.

To guarantee only microorganisms from the root canal were sampled, thorough decontamination procedures are necessary to ensure asepsis of the operative field. Hydrogen peroxide is a strong biocide (Linley \textit{et al.} 2012). Even at concentrations lower than 2.5 mM $\text{H}_2\text{O}_2$, DNA is damaged via Fenton-like reactions. At commercially available concentrations, amino acids and proteins are damaged inhibiting cell metabolism. Also lipids and cell membranes are damaged, leading to leakage of intracellular compounds and loss of cell integrity (Linley \textit{et al.} 2012). Still, 40\% of the samples had to be excluded because of contaminated sterility checks, which is a major limitation of the current study. Möller (1966) showed only 1.8 - 16.7\% of contamination after 30\% $\text{H}_2\text{O}_2$ and 10\% iodine tincture. Another study showed 19\% and 28\% contamination despite exhaustive decontamination procedures with 10\% iodine tincture or 2.5\% sodium hypochlorite in addition to 30\% $\text{H}_2\text{O}_2$ (Ng \textit{et al.} 2003). Samples of the tooth surface, rubberdam and liquid rubberdam were unsterile in >30\% of the samples, both when assessed by cultivation and DNA analysis (Ng \textit{et al.} 2003). As it seems impossible to sterilize the operative field on all occasions, control for sterility has to be routinely performed and positive controls warrant exclusion.

\begin{table}[h!]
\centering
\caption{Characteristics and identification of the 10 root canal isolates. The results for the different positive samples for bacterial spores and the subsequent analyses.}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Sample & Gram stain & Morphology & Oxygen requirements & API test identification & Chopped meat broth & Sequencing identification \\
\hline
sp5-1 & + & coccus & - & & & \\
sp5-2 & + & coccus & - & & & \\
sp5-3 & + & coccus & - & & & \\
sp5-4 & + & coccus & - & & & \\
sp8-2 & + & bacillus & - & \textit{Clostridium bifermentans} & + & Similar to sp8-3 \\
sp8-3 & + & bacillus & - & \textit{Clostridium bifermentans} & + & \textit{Propionibacterium acnes} \\
sp15-1 & + & bacillus & + & unclear & +++ & \textit{Propionibacterium avidum} \\
sp15-2 & + & bacillus & - & unclear & + & Similar to sp8-3 \\
sp15-4 & + & bacillus & - & unclear & + & Similar to sp8-3 \\
sp15-5 & + & bacillus & - & \textit{Propionibacterium acnes} or \textit{Clostridium sp.} & + & Similar to sp8-3 \\
\hline
\end{tabular}
\end{table}

of the root canal sample. Additionally, a second disinfection step could be applied when preparing the operative field for sampling.

Another prerequisite for performing this study was the ability to locate all microorganisms situated within the root canal infection. As apical periodontitis cannot occur without pulp necrosis and subsequent microbial infection (Kakehashi et al. 1965), every tooth with apical periodontitis contains a certain number of microorganisms. However, not in all cases these can be recovered clinically using current sampling techniques. When sampling the infection using paper points, not all extremities of the root canal system are reached. When the whole tooth is analysed, all extremities are included within the analysis and this allows microbial detection in all samples (Akpata 1974). A drawback of this method is that the tooth has to be extracted, which is undesirable from a clinical point of view. The analytical technique also has a threshold of detection. Cultivation is usually less sensitive than DNA analysis, and similarly non-targeted techniques are more likely to detect any microorganism than targeted techniques (Zambon & Haraszthy 1995). Besides, not all bacteria are cultivable or some species are not-yet-cultivable (Munson et al. 2002). Nonetheless, the technique employed in the current study relied on the difference between cultivability of spores and other bacteria.

Moreover, this study hypothesized that spores are present in the root canal. This assumption is based on the previous detection of bacteria capable of sporulation within root canal infections (Özok et al. 2012). However, sporulation only occurs in environments where essential nutrients are lacking or which suddenly turn threatening, such as during root canal treatment (Madigan et al. 1997). Nutrients may be available via coronal leakage, from pulp residues, microbial flora and inflammatory exudates, although in lesser amounts than in the oral cavity. Another consideration is that both the space and available nutrients are severely limited in persistent or reinfections. Hitherto, nutrient availability within root canal infections is variable and there is no consensus on whether it is rich or depleted. Unfortunately, all samples from previously treated canals were discarded, so stimulation of sporulation following root canal treatment could not be verified. Thus, the current study was unable to determine whether the root canal system is a favourable environment for sporulation and the question remains unresolved so far.

Bacterial spores were not detected within the root canal infections of this study. However, two propionibacteria species were detected. *P. acnes* and *P. avidum* are Gram-positive, facultatively anaerobic bacilli (Marples & McGinley 1974). Both are skin commensals, but *P. acnes* dominates the sebum rich areas, whereas *P. avidum* resides mainly in moist areas, such as the axilla (Marples & McGinley 1974). Propionibacteria have been isolated from primary root canal infections (Kantz
& Henry 1974; Rolph et al. 2001; Sassone et al. 2007; Vianna et al. 2008), persistent root canal infections (Sundqvist et al. 1998; Rolph et al. 2001; Niazi et al. 2010) and apical granulomas (Signoretti et al. 2013). These bacteria are often considered contaminants but Niazi et al. (2010) have shown that propionibacteria from root canal infection isolates are significantly different from skin isolates. However, as these bacteria are non-spore-forming and with the methods applied within this study, it is unusual that they pass the 80°C incubation step. Although heat resistance has been seen before in *Propionibacterium cyclohexanicum* (Kusano et al. 1997) and some streptococci (Hall 1925), it relies on another mechanism than bacterial sporulation, which was the focus of the present study.

The current methodology and sample size restricted the possible outcome of this pilot study, which can be circumvented by using more sensitive techniques. The sample volume and the extent to which the entire root canal system is sampled can be increased by pulverizing and analysing the whole root (Akpata 1974; Özok et al. 2012). Instead of cultivating live bacteria, other analytical techniques may target the specific spore phenotype using functional gene description, protein expression and metabolite secretion. Quantitative polymerase chain reaction of the functional sporulation gene spo0A could detect as few as 100 gene copies and $10^4$ gene copies per gram of environmental sediment (Bueche et al. 2013). However, DNA extraction from spores is particularly tough as they are hard to lyse, which decreases the chances of spore detection. Instead, immunoassays use antibodies to detect spore antigens on the outer surface. Current research is focusing on specific spores, such as *Bacillus anthracis*, with detection limits as low as 30 spores (Morel et al. 2012). This assay was designed to target one specific species, but an assay could be developed to target universal spore antigens. Alternatively, dipicolinic acid is a metabolite that is very specific to spores and contributes 5 - 15% to its weight. A complex is formed together with lanthanide elements, which can be detected rapidly using its fluorescence (Hindle & Hall 1999). The detection limit is still around $10^4$ *Bacillus* spores, though. Thus, detection of spores present at low numbers in small samples is still challenging, but techniques are evolving rapidly.

**CONCLUSION**

Within the limitations of this study, no endospores could be detected within root canal infections. More sensitive techniques should be applied on a larger sample size to definitively dismiss the presence of spores within root canal infections. If bacterial spores are present within their own separate niche, this calls for adjunctive treatment measures to efficiently clean the root canal system and support resolution of the periapical inflammation.
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AUTHOR CONTRIBUTIONS
Conceived and designed the study: IFP, MAH, WC. Performed the study: IFP and MAH. Analysed the data: ARO, IFP, MAH and WC. Drafted the manuscript: IFP. Critically revised the manuscript: ARO, MAH and WC. All authors accepted the final version of the manuscript.