Identification of oral spirochetes at the species-level and their association with other bacteria in endodontic infections

연세대학교 대학원

치의학과

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지도 이 찬 영 교수

이 논문을 박사 학위논문으로 제출함

2000년 12월 일

연세대학교 대학원

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정 일 영

정일영의 박사 학위논문을 인준함

심사위원 _____ 인

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연세대학교 대학원

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감사의 글

본 논문이 완성되기까지 지도와 격려를 아끼지 않으신 이찬영 교수님께 무한한 감사를 드립니다. 또한 본 연구의 부족함을 지적하여 보다 좋은 논문이 될 수 있도록 많은 충고와 조언을 해주신 이승종 교수님, 윤태철 교수님, 유윤정 교수님, 금기연 교수님 그리고 최봉규 교수님께도 감사를 드립니다. 본 실험을 진행할 때 많은 도움을 준 황대선 선생을 비롯한 우리 보존과 의국원들에게도 진심으로 감사드립니다.

아울러 이 조그만 논문을 완성시키기 위해 부모님과 저의 처 그리고 장인, 장모님을 비롯한 가족들이 보여준 헌신적인 사랑에 감사 드립니다. 특히 논문 핑계로 아버지 역할을 제대로 하지 못해 우리 의헌에게 다시 한 번 미안함을 느끼며 마지막으로 이렇게 여러 사람의 수고와 희생으로 완성된 저의 논문이 많은 사람들에게 도움이 되기를 간절히 바라며 감사의 글을 띄웁니다.

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저자 씀

차 례

표 차례

Abstract

Identification of oral spirochetes at the species-level and their association with other bacteria in endodontic infections

Department of Dentistry, Graduate School, Yonsei University

Directed by Prof. Chan-Young Lee, *D.D.S. Ph.D.*

Recent molecular approaches have revealed that fastidious organisms such as *Bacteroides forsythus* and oral treponemes are frequently found in the root canals with apical periodontitis. The purpose of this study was to identify the isolates of oral spirochetes at the species-level in endodontic infections and determine their association with *Bacteroides forsythus* and *Porphyromonas gingivalis*. Seventy-nine teeth with apical periodontitis were selected for this study. After sampling from the root canals aseptically, PCR amplification for the 16S rRNA gene was performed with eubacterial universal primers. Subsequently, dot-blot hybridization was performed with 8 species-specific oligonucleotide probes. The microbial associations were analyzed using the odds ratio.

The most frequently found species was *P. gingivalis* (27.4%), followed by *Treponema maltophilum* (26%), *B. forsythus (16.4%)* and *Treponema socranskii (2.7%)*. The other treponemes including *Treponema denticola* were not detected in our samples.

Significant microbial associations were identified between *T. maltophilum*, *B. forsythus* and *P. gingivalis* by utilizing analysis with the odds ratio. The results indicate that *T. maltophilum* should be included in the etiologic studies of endodontic diseases.

Keywords : Spirochetes, Endodontic infection, Dot-blot hybridization, PCR

Identification of oral spirochetes at the species-level and their association with other bacteria in endodontic infections

Department of Dentistry, Graduate School, Yonsei University

Directed by Prof. Chan-Young Lee, *D.D.S. Ph.D.*

Il-Young Jung, *D.D.S., M.S.*

I. Introduction

Apical periodontitis is attributed to a larger complex of bacteria (1,2). However, many investigators (2-4) have suggested that some species may have a key function in the development of the disease. In accordance with this, recent molecular genetic analyses on the root canal flora (5-7) have revealed some notable findings: both *Bacteroides forsythus* and oral treponemes, which were considered to be important pathogens in periodontal diseases (8,9), were frequently found in the infected root canals. These findings have not been observed by previous culture-based investigations (1-4, 10,11). Since frequent detection of these bacteria from the infected root canals implies that the anaerobes also play important roles in endodontic diseases in addition to periodontal diseases, it is necessary to investigate the etiologic roles of these bacteria in the root canal infections.

The first step in assessing the role of treponemes in endodontic

infections is to investigate the extent to which these bacteria are established in the root canals at the species-level. Some investigators (12-14) have observed various spirochetal morphotypes in the root canals with dark-field or electron microscopy, and Dahle et al. (15) performed a biochemical identification to characterize the isolates of spirochetes. However, these methods have some limitations in classifying the variants of oral spirochetes. To date, few studies have attempted to identify the endodontic isolates of spirochetes at the species-level. Recent 16S rRNA-based analyses have revealed an unexpected diversity of oral treponemes in the subgingival pocket (16, 17). Among these, 8 species have been identified and named (17): *Treponema denticola* (18), *Treponema vincentii* (19), *Treponema socranskii* (20), *Treponema pectinovorum* (21), *Treponema maltophilum* (22), *Treponema medium* (23), *Treponema amyloborum* (24) and *Treponema lecithinolyticum* (25). Since the 16S rRNA approach is thought to be one of the most useful tools in discriminating oral spirochetes, this method is appropriate in investigating the endodontic isolates.

Many investigators (8, 26, 27) have suggested that certain microbial complexes are more important in regulating the severity of infection than the presence of individual species. In the view of these associations, *B. forsythus* and *Porphyromonas gingivalis* have been noted in periodontal infections. Along with *T. denticola*, they are intimately related to each other, and the presence of their complexes is strongly correlated with the severity of periodontal disease (8). We could not find any complex associated with the symptomatic teeth in the previous study (7). However, we found that some species appear frequently as complexes in

the root canals as well as in the subgingival plaque.

The root canal flora is useful in investigating the microbial association because contamination is minimized and no normal flora is found. Previous studies (6, 8, 26, 27) on the microbial associations have mainly been focused on *T. denticola* among oral spirochetes. Considering the diversity of spirochetes in the subgingival pocket, it is necessary to reassess the microbial association by the species-specific probes.

The purpose of this study was (a) to identify the isolates of oral treponemes from the root canals at the species-level; and (b) to determine the association among the selected bacteria in the root canal flora. This work was performed by polymerase chain reaction (PCR) and dot-blot hybridization using oligonucleotides probes based on the 16S rRNA.

II. MATERIALS AND METHODS

Clinical samples. Seventy-nine patients treated at the Dental Hospital of Yonsei University were selected for this study. Their ages ranged from 11 to 71 years (mean 39). The selected teeth had single roots containing necrotic pulps and radiographic evidence of periapical bone resorption. None of these teeth had been administered either antibiotic medication within the preceding 3 months, nor periodontal pocket extended to the apical third of the tooth.

The sampling procedures outlined in a previous study (7) were used. Briefly, the teeth and rubber dam were disinfected with NaOCl and an access opening was made aseptically. If upon opening, a sterile paper point was then introduced and retained for 20 sec. Where a dry canal was identified, sterile water was introduced into the canal and mixed with the contents of the root canal using a file. A total of 3 paper points were obtained from each tooth. The paper points were immediately placed in 1 ㎖ of reduced transfer fluid and transferred to the laboratory.

Bacterial strains. Thirty-nine oral bacterial strains were chosen to evaluate the specificity of the oligonucleotides probes used in this study. The microorganisms included 25 strains from the American Type Culture Collection (ATCC, Rockville, MD) and 14 clinical isolates (Table 1).

Table 1. Oral bacterial strains employed in this study

Bacteria	ATCC No.	clinical isolate
Treponema denticola	33521	
Treponema pectinovorum	33768	
Treponema maltophilum	51939	
Treponema lecithinolyticum	700332	
Treponema vincentii	35580	
Treponema socranskii subsp. socranskii	35536	
Treponema socranskii subsp. buccale	35534	
Treponema amylovorum		700288
Treponema phagedenis		biotype Reiter
Bacteroides forsythus	43037	057/35-6
Porphyromonas gingivalis	33272	057/35-1
	33277	A7A1-28,
	53978	
Actinobacillus actinomycetemcomitans	29522	D56
	33384	
Fusobacterium nucleatum	49256	058/16-3a
	25586	058/16-3b
		058/16-3c
Peptostreptococcus micros	33270	057/35-5
Peptosteptococcus prevotii	14951	
Porphyromonas endodontalis	35406	
Prevotella intermedia	25611	
	49046	
Prevotella nigrescens	33536	D-96/3
		NCTC 9336
Actinomyces israelii		057/35-10
Actinomyces naeslundii	12104	
Campylobacter concisus		
057/34-4b		
Capnocytophaga gingivalis		060/16-2b
Lactobacillus casei	27216	
Streptococcus mutans	25175	

DNA extraction and amplication. Aliquots of each sample (500 μℓ) were centrifuged (Jouan, Saint-Herblain, France) at 13,000×g for 10 min. The resulting pellets were washed with 500 μℓ of phosphate buffered saline, and placed in 100 μℓ of lysis buffer (500mM Tris-HCl (pH 9.0), 20 mM EDTA, 10mM NaCl, 1% SDS). DNA extraction was performed with phenol (2 vol.): phenol (1 vol)/chloroform (1 vol): chloroform (2 vol.). One microliter of bulk DNA was used for PCR in a thermal cycler for 30 cycles of denaturation (1 min, 95℃), annealing (1min, 55℃), and extension (1min, 72℃). The broad-range eubacterial primers used for 16S rRNA gene amplification were TPU1 (5`AGA GTT TGA TCM TGG CTC AG-3: corresponding to positions 8 to 27 in *E.coli* 16S rRNA) and RTU3 (5-GWA TTA CCG CGG CKG CTG-3, which corresponded to positions 519 to 536 in *E. coli* 16S rRNA). PCR of the reference bacterial strains was performed with cell pellets collected from the cultures, and the PCR products of approximately 500 bp were confirmed by agarose gel electrophoresis.

Oligonucleotide probes. The oligonucleotide probes for hybridization were selected from the publication (28) or made from the sequences in the EMBL and GeneBank databases. Six species-specific probes were used to characterize the isolates of oral treponemes. Probes TDEN, TVIN, TSOC, TPEC, TMAL, and TLEC were synthesized to detect the known cultivable treponemes *T. denticola, T. vincentii, T. socranskii, T. pectinovorum, T. maltophilum* and *T. lecithinolyticum,* respectively. In addition, probes PGIN and BFOS were prepared to detect *P. gingivalis* and *B. forsythus,* respectively. The specificity of each probe was confirmed with the reference bacterial strains. The sequences of each

probe are presented in Table 2. The probes were labeled with digoxigenin (DIG)-ddUTP using a DIG oligonucleotide 3-end labeling kit (Boehringer Mannheim, Germany) according to the manufacturers instructions.

Table 2. Sequences of the selected oligonucleotides

Probes	Sequence (5'-3') of probes
TDEN	CATGACTACCGTCATCAAAGAAGC
TVIN	ATTGAGACTATTCGGTATTACCTGC
TSOC	CATTGCTGCCTGCCGCTCGACTTG
TPEC	CTCCAACTTATATGACCTTATCCG
TMAL	CTATTGTGCTTATTCATCAGGC
TLEC	CACTCTCAGAAAGGAGCAAGCTCC
BFOS	CGTATCTCATTTTATTCCCCTGTA
PGIN	TACTCGTATCGCCCGTTATTC

The probe TSOC could detect *T. socranskii* subsp. *socranskii* and *T. socranskii* subsp. *buccale*.

Dot-blot hybridization. After denaturation of the PCR products at 95℃ for 5 min, 2㎕ aliquots were blotted onto the nylon membrane (Hybond N, Amersham) and fixed by UV cross-linking for 3min (Ultraviolet crosslinker, UVP, Inc., Upland, CA, USA). After 30 minute-prehybridization at the probe-specific temperature in the hybridization buffer (5×SSC, 0.1% N-laurosylsarcosine, 0.02% SDS, 1%

blocking reagent), hybridization was performed in the same buffer for 1 h after the addition of a DIG-labeled oligonucleotide probe (50 pmol). Two stringent washes were done at the hybridization temperature for 30 min with a washing buffer (5×SSC, 0.1% SDS). After the addition of anti-DIG-alkaline phosphatase conjugates according to the manufacturers recommendations. the DIG-labeled probes were detected with the chemiluminiescent substrate CSPD (Boehringer Mannheim, Germany). The X-ray films were exposed to the membranes for 2 to 5 h.

For hybridization using another probe, the membranes were washed twice in a stripping buffer (0.1M NaOH, 0.1% SDS) at 37℃ for 15 min, and equilibrated with 2×SSC. Subsequently, hybridization was performed as above.

Statistical Analysis. To determine the associations between the species, statistical analysis outlined in the previous investigation was performed (7). Briefly, the odds ratios were computed from logistic regression analysis (SAS 6.12 program). In this analysis, the presence or absence of individual species was used as a response variable and the other species as an independent variable, respectively. When the odds ratio was above 2.0 and the confidence range did not include 1.0, the association was concluded to be significant.

III. Results

A total of 73-PCR products were obtained from 79 teeth. Among these products, 43 samples did not react with any of the probes employed in this study. Hybridization signals were observed in remaining 30 samples, and 4 species were detected. The number of individual species detected in this study is shown in Table 3. Under the conditions of our investigation, no cross hybridization was observed except for the TMAL probe, which showed a hybridization signal with both *T. lecithinolyticum* and *T. maltophilum*. Since the TLEC probe did not react with any of the samples in this study, we regarded the isolates that reacted with the TMAL probe as *T. maltophilum*. Among the oral treponemes investigated, *T. maltophilum* (19 samples) and *T. socranskii* (2 samples) were the only species that were observed. The other treponemes investigated were not detected in this study.

P. gingivalis and *B. forsythus* were found in 20 and 12 samples, respectively. Due to the limited number of *T. socranskii*, the microbial association was only evaluated on *T. maltophilum*, *P. gingivalis* and *B. forsythus* (Table 4). Significant associations between the 3 species were identified and are summarized in Table 5.

Table 3. Microorganisms detected from root canals

Microbial species	No. of samples
T. maltophilum	19
T. socranskii	2
P. gingivalis	20
B. forsythus	12

Table 4. The observed frequencies of the 3 species individually and in different combinations.

1st species	2nd species	absence of both	1st only	2nd only	presence of both
T. maltophilum	*P. gingivalis*	*44*	*9*	*10*	*10*
P. gingivalis	*B. forsythus*	50	11	3	9
B. forsythus	*T. maltophilum*	49	5	12	7

Table 5. Odds ratios of associations among the selected organisms

Species	Odds ratio	95% Confidence Intervals lower	upper
T. maltophilum / P. gingivalis	4.889	1.575	15.171
P. gingivalis / B. forsythus	13.636	3.165	58.749
T. maltophilum / B. forsythus	5.717	1.543	21.183

The significance of associations between 3 species was tested using the logistic regression analysis. All of them were statistically significant.

IV. Discussion

Although Brown et al. (12) described the presence of spirochetes in their pulp samples about 40 years ago, the role of these organisms in root canal infections has been underestimated. Some authors (29) have suggested that the presence or absence of oral spirochetes might be an aid in differentiating endodontic and periodontal abscesses. It is obvious that spirochetes are found more frequently in the subgingival plaque than in the root canals. However, these findings may not be relevant when we speak of the effects of the oral treponemes in endodontic infections. Recent molecular genetic analyses (6, 7) have revealed that spirochetes are found frequently in the infected root canals, which concurs with this study. With the exception of Molven et als study (13), the incidence of treponemes in this study was higher than that of the previous studies (6, 15). It is likely that this difference is due to the methodology used. It is generally accepted that PCR has a higher sensitivity than “Checkerboard” and light microscopy-based methods (6, 28).

The intriguing feature about the present study is that most of the oral treponemes in the root canals were *T. maltophilum*, which is a representative of the phylogenic group IV treponemes and was recently isolated by Wyss et al (22). *T. maltophilum* is a small motile treponeme and has two periplasmic flagella. The organism was frequently isolated from patients with rapidly progressive periodontitis (28). The virulence factors of the bacteria are currently under investigation. Heuner et al. (30, 31) proposed that the motility of *T. maltophilum*, caused by rotation of the periplasmic flagella, might contribute significantly to its

pathogenicity. Although we found that *T. maltophilum* is present in many root canals, we are not certain whether the cell has a key role in mediating the destructive effects of an endodontic infection. Since the bacterium was strongly postulated as an important pathogen in periodontitis, investigating its role in endodontic pathology is worthwhile. Siqueira et al. (6) observed *T. denticola* in root canals, and it was, therefore, unexpected that *T. denticola* was not detected in this study. Dahle et al. (15) found that their isolates differed from *T. denticola, T. socranskii subspecies,* and *T. pectinovorum*. They believed that their endodontic isolates represented two new species. However, it is difficult to compare their study with ours because of different methodologies used. Siqueira et al. (6) used DNA-DNA hybridization to detect the anaerobes from the root canals. Moreover, they did not include a specific probe for *T. maltophilum*. The checkerboard method has been widely used to investigate the microbial ecology. However, one concern with this method is the specificity of the DNA probes employed. In addition, Socransky et al. (8) also mentioned the possibility of cross-reactions within genera. We used oligonucleotide probes based on 16S rRNA sequences to discriminate species, which are less likely to cross-react (32). However, we cannot suggest that *T. denticola* does not exist in root canals. Nevertheless, it seems reasonable to conclude that other spirochetal species such as *T. maltophilum* prevail in root canal systems. Although the TMAL probe used in this study to detect *T. maltophilum* cross-reacted with *T. lecithinolyticum*, the closest cultivable relative of *T. maltophilum* (25), it did not exhibit cross-reactions with any of the other spirochetes investigated. Our results showed that the organisms regarded as *T.*

maltophilum were not *T. lecithinolyticum*. However, the possibility could exist that they were a previously unclassified species of the phylogenic group IV treponemes.

It was interesting to compare the distribution of oral spirochetes in the root canals with the subgingival plaque samples. It appears that for oral spirochetes, the periodontal pocket is a more favorable refuge than the root canal. Using a similar methodology, Moter et al. (28) revealed that *T. socranskii* (96.2%) was most frequently found in the deep periodontal pocket, followed by *T. maltophilum* (66.0%), *T. denticola* (62.3%), and *T. vincentii* (20.8%). Although spirochetes in the root canals were not so diverse as in the periodontal pockets, they are similar in that *T. socranskii* and *T. maltophilum* were more frequently found than the other spirochetes. Many investigators have reported that *T. denticola* is most frequently associated with chronic periodontal disease (33, 34). However, as Moter et al. (28) mentioned, it remains to be determined whether this organism is of etiologic relevance or merely the most easily cultured organism. In their study, *T. maltophilum* was detected much more often in disease sites than in the respective healthy sites. Dewhirst et al. (17) also corroborated this finding. As a consequence of these investigations (28) and our own, *T. maltophilum* should be included in any etiologic study of endodontic disease as well as periodontitis.

In terms of microbial associations, some authors (8) have shown that *P. gingivalis*, *B. forsythus*, and *T. denticola* have intimate associations with each other. Since *T. denticola* could not be detected in this study, such microbial associations were analyzed by substituting *T. maltophilum* for *T. denticola*. The results showed that *T. maltophilum* frequently appeared

with *P. gingivalis* and *B. forsythus*. When one considers that the root canal system is an ideal place in investigating the microbial ecology, these results appear to be very meaningful in understanding the microbial associations. Accordingly, previous investigations (6-8) and this study have indicated that the role of oral treponemes should be considered in the view of microbial complex as well as the individual species. Socransky et al. (8) suggested that these microbial complexes are intimately related to the clinical parameters. But, we did not perform these analyses during this study for the following reasons; it appears that the severity of infection relies not only on the presence of bacteria, but also on the quantity of pathogens present. The PCR, which we used, was inappropriate for the quantitative determination of the pathogens. In addition, spirochetal diseases have some important characteristics, such as their chronic and episodic nature. As a consequence, further detailed investigation combining both quantitative and qualitative measurements is necessary in understanding the role of these microbial complexes in root canal infections.

In conclusion, among the oral spirochetes investigated, *T. maltophilum* was the most frequently found. In addition, this pathogen was significantly associated with *P. gingivalis* and *B. forsythus*. These findings provide some evidence for the potential effects of treponemes in endodontic infections as well as in periodontal diseases.

V. Conclusion

The aim of this study was to identify the isolates of oral spirochetes at the species-level in endodontic infections and determine their association with *Bacteroides forsythus* and *Porphyromonas gingivalis*. Seventy-nine teeth with apical periodontitis were selected for this study. After sampling from the root canals aseptically, PCR amplification for the 16S rRNA gene was performed with eubacterial universal primers. Subsequently, dot-blot hybridization was performed with 8 species-specific oligonucleotide probes. The microbial associations were analyzed using the odds ratio. The results were as follows;

1. The most frequently found species was *P. gingivalis* (27.4%), followed by *Treponema maltophilum* (26%), *B. forsythus (16.4%)* and *Treponema socranskii (2.7%)*. The other treponemes including *Treponema denticola* were not detected in our samples.
2. Significant microbial associations were identified between *T. maltophilum*, *B. forsythus* and *P. gingivalis* by utilizing analysis with the odds ratio.

The results indicate that *T. maltophilum* should be included in the etiologic studies of endodontic diseases.

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국문요약

근관 감염에서 구강 스피로헤타의 분포 및 다른 세균과의 상관 관계

연세대학교 대학원 치의학과
(지도교수 이 찬 영)
정 일 영

최근 근관 내 미생물을 연구하는데 있어서 분자생물학적 연구 방법은 *Bacteroides forsythus*나 구강스피로헤타 등이 치근단 치주염을 가진 근관 내에서 자주 발견됨을 보여주었다. 본 연구의 목적은 근관 감염에서 구강스피로헤타의 분포를 종 단계에서 확인해보고 이들 구강 스피로헤타와 *B. forsythus* 그리고 *Porphyromonas gingivalis* 와의 상관관계를 알아보는 것이다. 치근단 치주염을 가지는 79개의 치아가 선택되었다. 근관 내 세균을 가능한 오염되지 않게 채취한 후에 16S rRNA 유전자를 이용하여 중합효소반응과 점상블롯보합 결합법을 시행하였다. 본 연구에 사용된 소식자는 6종 구강스피로헤타와 *B. forsythus* 그리고 *P. gingivalis*를 검출 할 수 있다. 각각의 세균간의 상관관계는 비차비를 이용하여 평가하였다.

본 연구에서 가장 많이 발견된 세균은 *P. gingivalis* (27.4%) 였고 *Treponema maltophilum* (26%), *B. forsythus (16.4%)* 그리고 *Treponema socranskii (2.7%)* 순으로 발견되었다. *Treponema denticola*를 포함하여 다른 구강스피로헤타 종들은 본 연구에서 발견되지 않았다.

비차비를 이용한 세균 종간의 상관관계는 *T. maltophilum*, *B. forsythus* 그리고 *P. gingivalis*에 대해서 행해졌는데 이들 세균종들은 상호간에 밀접한 연관성을 나타내었다. 본 연구 결과는 근관내 감염에 의한 치근단 질환의 발생을 연구하는데 있어 *T. maltophilum*이 포함되어야 한다는 것을 보여주었다.

핵심되는 말: 구강스피로헤타, 근관내 감염, 점상블롯보합 결합법, 중합효소반응