Oral Microbiology Immunology 2005: 20: 253–258
Printed in Singapore. All rights reserved

Oral Microbiology and Immunology

Short communication

Microarrays complement culture methods for identification of bacteria in endodontic infections

M. E. Vianna[1,2], H.-P. Horz[2], B. P. F. A. Gomes[1], G. Conrads[2]
[1]Endodontic Area, Department of Restorative Dentistry, Piracicaba Dental School, State University of Campinas, Piracicaba, SP, Brazil, [2]Division of Oral Microbiology and Immunology, Department of Operative and Preventive Dentistry & Periodontology, and Institute of Medical Microbiology, University Hospital, RWTH Aachen, Germany

Vianna ME, Horz H-P, Gomes BPFA, Conrads G. Microarrays complement culture methods for identification of bacteria in endodontic infections. Oral Microbiol Immunol 2005: 20: 253–258. © Blackwell Munksgaard, 2005.

The aim of this study was to investigate the microbial composition of necrotic root canals using culture methods and microarray technology. Twenty uniradicular teeth with radiographic evidence of periapical bone loss and with no previous endodontic treatment were selected for this study. For molecular diagnosis a DNA chip with 20 different species-specific, 16S-rDNA-directed catcher probes was used. The microorganisms most frequently detected by the DNA chip were: *Micromonas micros*, *Fusobacterium nucleatum* ssp., *Tannerella forsythia*, *Treponema denticola*, *Veillonella parvula*, *Eubacterium nodatum*, *Porphyromonas gingivalis*, *Actinomyces odontolyticus*, and *Streptococcus constellatus*. As expected, additional important bacterial taxa were found by culture analysis, but microorganisms such as *T. forsythia* and *T. denticola* could not be identified. In conclusion, microarrays may provide significant additional information regarding the endodontic microbiota by detecting bacterial species that are otherwise difficult or impossible to culture.

Key words: 16S rRNA gene; endodontic infection; microarrays; microbial identification

Georg Conrads, Division of Oral Microbiology and Immunology, University Hospital, RWTH Aachen Pauwelsstrasse 30, D-52057 Aachen, Germany
Tel.: +49 2418088448;
Fax: +49 2418082483;
e-mail: gconrads@ukaachen.de
Accepted for publication February 22, 2005

Opportunistic pathogenic bacteria that colonize the oral cavity have been implicated in endodontic infections and periradicular lesions (13). Several mainly culture-based approaches have been made to identify bacteria in infected root canals (1, 5, 8, 10). The prevalence of certain recognized oral pathogens could have often been under-estimated because culture-based techniques may fail to grow fastidious anaerobic microorganisms such as spirochetes (22) or *Tannerella forsythia*, formerly known as *Bacteroides forsythus* (5).

Therefore, the use of nucleic-acid based techniques may lead to a more comprehensive picture of the microbiota associated with necrotic teeth. However, testing a sample species by species by for example using individual PCR primers or DNA probes can be very time consuming. An alternative approach is screening for multiplex species identification using microarray technology. Microarrays were originally introduced for differential expression profiling in both eukaryotic and prokaryotic cells (2–4, 27). They have also been applied to support bacterial identification, especially in mixed infections and microbial ecology (11).

The ParoCheck®-chip (Greiner, Bio-One GmbH, Frickenhausen, Germany) is a commercially available microarray for the detection of up to 20 different oral bacterial species. It was originally developed to support the clinical diagnosis of chronic and aggressive forms of periodontitis.

The aim of the present study was to investigate the principal applicability of microarrays in general and the ParoCheck-chip® (Greiner-Bio-One) in particular for analyzing the microbial composition of necrotic teeth with apical periodontitis.

Material and methods

Twenty patients at the Piracicaba Dental School (PDS) for root canal treatment, who were otherwise healthy and had not received antibiotic treatment during the previous 3 months, were selected for this study. The age of the patients ranged from 19 to 63 years. The selected teeth (one tooth per patient) were uniradicular, presented necrotic pulp tissues, and showed radiographic evidence of apical periodontitis. All teeth were asymptomatic. A detailed medical and dental history was obtained from each patient. The Human Volunteers Research and Ethics Committee of the Dental School of Piracicaba approved a protocol describing the

specimen collection for this investigation, and all patients signed an informed consent to participate in the study.

The teeth were isolated with a rubber dam. The crown and the surrounding rubber dam were disinfected with 30% H_2O_2 (v/v) for 30 s followed by 2.5% NaOCl for additional 30 s. Subsequently, 5% sodium thiosulfate was used to neutralize the disinfectant agents (12). An access cavity was prepared with sterile high-speed diamond burs under irrigation with sterile saline. Before entering the pulp chamber, the access cavity was disinfected with the same protocol as mentioned above. The sterility was checked by taking a swab sample of the cavity surface and streaking on blood agar plates, but also by the same universal polymerase chain reaction (PCR) described below (detection limit 500 cells). All subsequent procedures were performed aseptically. The pulp chamber was accessed with sterile burs refrigerated in saline. The samples were collected with four sterile paper points, which were consecutively placed into the canal to the total length calculated from the preoperative radiograph. Afterwards, the four paper points per root canal were pooled in a sterile tube containing 1 ml RTF (25). The samples were split 1 : 1. One half was immediately processed for culture analysis at the Endodontic Microbiology Laboratory (PDS, Brazil), while the other was transported on dry ice by an overnight delivery service to the Division of Oral Microbiology (University Hospital, RWTH Aachen, Germany) for subsequent microarray analysis.

Inside the anaerobic chamber the transport media were shaken thoroughly in a mixer for 60 s (Vortex, Marconi, São Paulo, SP, Brazil). Serial 10-fold dilutions were made up to 10^{-4} in tubes containing Fastidious Anaerobe Broth (FAB, Laboratory M, Bury, UK). Fifty µl of the serial dilutions 10^{-2}, 10^{-3}, and 10^{-4} were plated using sterile plastic spreaders onto Fastidious Anaerobe Agar (FAA, Laboratory M, Bury, UK) supplemented with 5% defibrinated sheep blood, 5 mg/l hemin, and 1 mg/l vitamin K_1, in order to nonselectively cultivate obligate and facultative anaerobes. Supplemented FAA was also used as selective culture medium by adding:

- 1 mg/l nalidixic acid and 0.5 mg/l vancomycin, to select gram-negative anaerobic bacteria;
- 7.5 mg/l kanamycin plus 0.5 mg/l vancomycin to select black-pigmented *Prevotella* and *Porphyromonas* species;
- 7.5 mg/l neomycin for clostridia and other anaerobes;
- 1 mg/l nalidixic acid for gram-positive anaerobes and *Actinomyces* spp.

The plates were incubated at 37°C in an anaerobic atmosphere (80% N_2, 10% CO_2, 10% H_2) for up to 14 days to permit detection of very slow growing strains. Using these dilutions, the detection level is $\geq 2 \times 10^3$ colony forming units (CFU)/ml.

The same dilutions were plated onto BHI agar plates (Brain Heart Infusion agar, Oxoid, Basingstoke, UK), supplemented with 5% sheep blood, in order to allow the growth of aerobic (37°C, air) or facultative anaerobic (37°C, 10% CO_2) microorganisms. Sabouraud-Dextrose agar (Oxoid), supplemented with 100 mg/l chloramphenicol (Medley, Campinas, SP, Brazil), was used to grow yeasts (37°C, air).

Preliminary characterization of microbial species was based on the colony features (size, color, shape, surface, texture, consistency, brightness, and hemolysis) as seen under a stereomicroscope (Lambda Let 2, Atto instruments CO, Hong Kong). Isolates were subcultured, gram-stained, tested for catalase-production, and their gaseous requirements were analyzed by incubation for 2 days aerobically and anaerobically. Based on this

Table 1. Culturing and microarray identification of microorganisms from primary endodontic infections

	Species detected by culture	Species detected by DNA chip
1	*A. meyeri, G. morbillorum, P. acnes, P. propionicum*	*M. micros, P. gingivalis, T. forsythia, T. denticola*
2	*A. odontolyticus, F. nucleatum, G. morbillorum, E. lentum, P. propionicum*	*C. gracilis, F. nucleatum, M. micros, S. constellatus, V. parvula*
3	*B. fragilis, E. lentum, F. nucleatum, G. morbillorum, P. acnes*	*F. nucleatum, T. forsythia*
4	*A. viscosus, A. naeslundii, G. morbillorum, S. mitis*	*A. odontolyticus, F. nucleatum, S. mitis, S. gordonii, S. constellatus, V. parvula*
5	*E. lentum, E. faecalis, F. nucleatum, H. aphrophilus, Micrococcus* spp., *P. acnes*	*T. denticola, T. forsythia*
6	*Actinomyces* spp., *E. faecalis, L. lactis*	Negative
7	*B. cereus, H. haemolyticus, Micrococcus* spp., *P. loescheii, P. oralis*	*A. odontolyticus*
8	*F. nucleatum, P. melaninogenica, Veillonella* spp.	*F. nucleatum, T. forsythia*
9	*A. radicidentis**, *Corynebacterium* spp.*, *P. acidopropionici**	Negative
10	*Rothia dentocariosa**	*M. micros*
11	Negative	*E. nodatum, M. micros, T. denticola, T. forsythia*
12	*A. calcoaceticus**, *A. naeslundii**, *S. salivarius**	Negative
13	*S. mitis**, *unidentified Streptococcaceae* spp.*	*E. nodatum, T. denticola, T. forsythia*
14	*Peptosteptococcus prevotii, P. corporis*	*M. micros*
15	*C. albicans, S. mutans**	*A. odontolyticus, E. nodatum, F. nucleatum, M. micros, S. constellatus, S. gordonii, V. parvula*
16	*A. baumanii*	*A. odontolyticus, E. nodatum, F. nucleatum, M. micros, P. gingivalis, S. constellatus, T. denticola, T. forsythia, V. parvula*
17	*A. viscosus, Clostridium* spp., *E. lentum, F. nucleatum, G. morbillorum, P. gingivalis, P. loescheii*	*E. nodatum, F. nucleatum, P. gingivalis, T. denticola, T. forsythia, V. parvula*
18	*Unidentified oral Streptococcaceae* spp.*	*F. nucleatum, M. micros, P. gingivalis*
19	*E. faecalis, C. albicans*	*M. micros*
20	*E. faecalis*	*M. micros*

*Identification of these isolates was supported by partial 16S rDNA gene sequencing (800/1500 bp). Underlined: species detected in the particular sample by both methods.

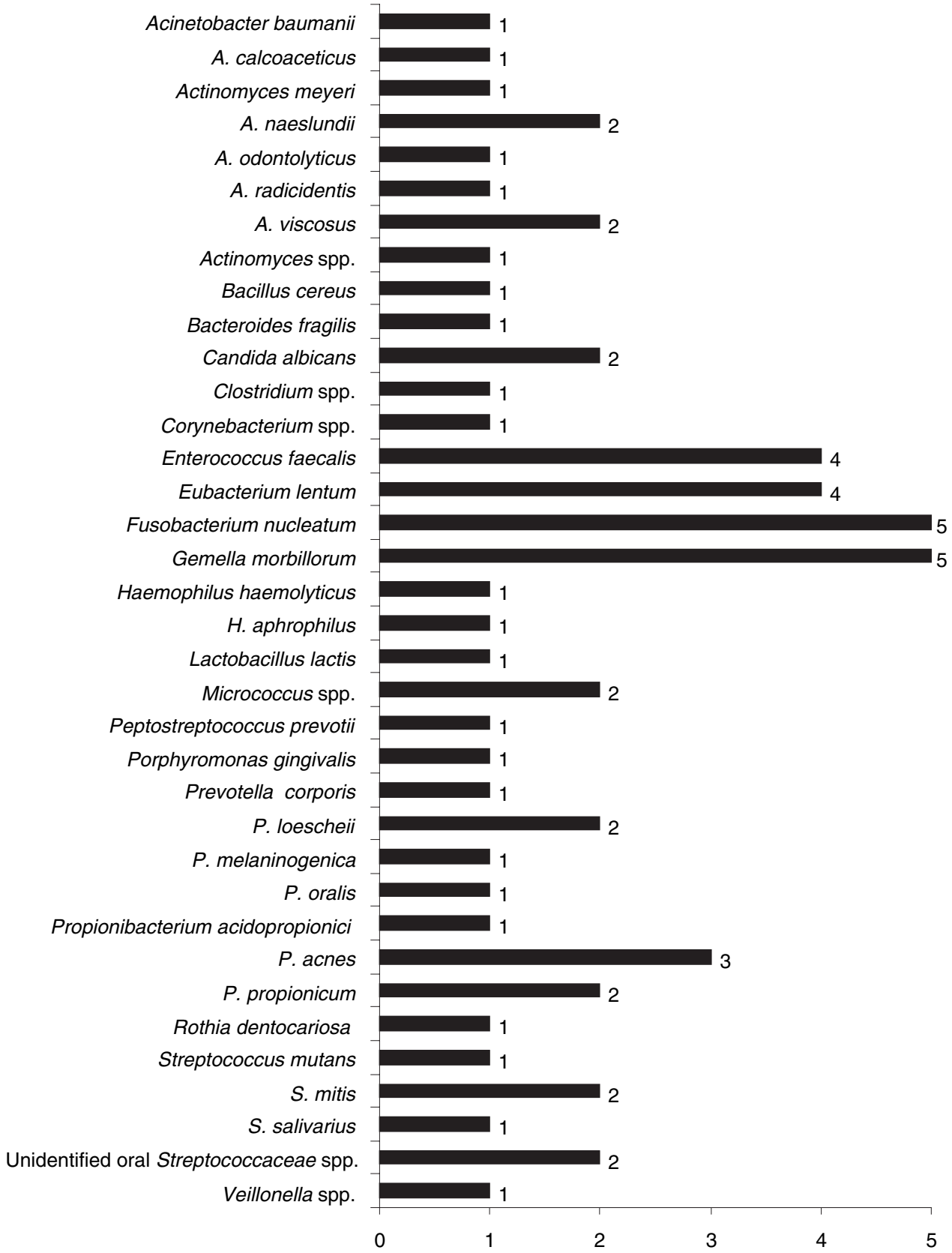

Fig. 1. Frequency of isolates from 20 necrotic root canals (culture).

information it was possible to select appropriate procedures for identification of isolates by commercial biochemical test panels (Rapid ID 32 A, API Staph, Rapid ID 32Strep, API C Aux [BioMérieux SA, Marcy-l'Etoile, France] and RapID ANA II System, RapID NH System [Innovative Diagnostic Systems Inc., Atlanta, GA]).

When the biochemical result was ambiguous, partial sequencing of the 16S rDNA was performed according to standard procedures using the following oligonucleotides as PCR and sequencing primers: forward primer 5′-AGA GTT TGA TCC TGG CTC AG-3′, reversed primer 5′-GGT TAC CTT GTT ACG ACT T-3′, as previously described (5).

For each sample, DNA was extracted and purified with the Qiamp DNA Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's instructions. The DNA concentration was measured at A_{260} and the purity calculated (A_{260}/A_{280}) (Gene Quant II, Pharmacia Biotech, Cambridge, UK).

The PCR amplification was carried out in a volume of 20 µl containing 17.5 µl Master Mix (delivered with the ParoCheck® kit, containing NTPs, and a forward as well as a 5′ end Cy5-labeled reversed primer, universally targeting 16S rDNA, Greiner Bio-One), 2.0 µl sample DNA, and 0.5 µl Taq DNA Polymerase (1 unit). The amplification was performed using a thermocycler (PCR Express, Hybaid Limited, Middlesex, England) and the following temperature profile and cycles: predenaturation 94°C for 1 min; 45 cycles: 95°C for 20 s, stringent annealing at 60°C for 20 s, 72°C for 30 s; final elongation: 72°C for 1 min. Negative controls (sterility check, without template) and positive controls (100 ng *Prevotella nigrescens* DNA) were included. By using 45 cycles, a detection limit of between 100 and 500 cells was reached.

Hybridization was performed according to the instructions of the ParoCheck® manufacturer (Greiner Bio-One). Each slide was incubated at 60°C in a container with a saturated humid environment for at least 5 min. Afterwards, 20 µl of hybridization buffer and 2 µl of the target PCR product were mixed at room temperature and 15 µl of this solution was transferred onto the slide surface and overlaid with a coverslip (25 × 25 mm). The slide incubation was continued for an additional 10 min. The coverslip was removed and the slide treated with a buffer system (supplied with the ParoCheck® kit) according to the manufacturer's instructions. The surface was dried by air spray. Results were automatically generated using a scanner (Axon 4100 A, Axon Instruments Inc., Union City, CA) and the PAROREPORT software (supplied with the ParoCheck® Kit, based on GenePix™, Axon Instruments). The following species can be identified by the ParoCheck® microarray detection system: *Actinobacillus actinomycetemcomitans, Actinomyces odontolyticus, Actinomyces viscosus, Campylobacter concisus, Campylobacter gracilis, Campylobacter rectus, Capnocytophaga gingivalis, Eikenella corrodens, Eubacterium nodatum, Fusobacterium nucleatum, Peptostreptococcus micros* (now called *Micromonas micros), Porphyromonas gingivalis, Prevotella intermedia, P. nigrescens, Streptococcus constellatus, Streptococcus gordonii, Streptococcus mitis, T. forsythia, Treponema denticola,* and *Veillonella parvula.*

Using culture techniques, 95% of the samples (19/20) were positive for at least one microbial species; only one root canal (sample no. 11) had no growth. Individual root canals yielded a maximum of seven species. In total, 61 isolates belonging to 35 different bacterial species and to *Candida albicans* were obtained (Table 1, column 1).

The microorganisms most frequently found by culture were *F. nucleatum* (n=5), *Gemella morbillorum* (n=5), *Eubacterium lentum* (n=4), and *Enterococcus faecalis* (n=4) (Fig. 1).

In contrast, by microarray analysis using the ParoCheck® kit based on a maximum of 20 selected bacterial species, 85% of the samples (17/20) were positive. A total of 58 identifications were made, belonging to 12 different bacterial species. Individual root canals yielded a maximum of nine bacterial species. Three samples were negative for all 20 species tested (Table 1, column 2). The result of one representative microarray is demonstrated in Fig. 2.

The microorganisms most frequently detected by the DNA chip were: *M. micros* (n=10), *F. nucleatum* ssp. (n=8), *T. forsythia* (n=8), and *T. denticola* (n=6) (Fig. 3).

In this study, we undertook a comparative investigation of the microflora of 20 infected and untreated root canals. To optimize the recovery of anaerobic species, we used prereduced nutritious media and incubated under anaerobic conditions for a prolonged period to ensure the recovery of slow-growing species. To support identification of isolates, comparative sequence analysis of 16S rDNA was used in those cases where biochemical characterization did not give clear results. 16S rDNA analysis allowed the identification of species such as *Actinomyces radicidentis* and *Rothia dentocariosa.* Interestingly, by 16S rDNA analysis, two isolates from samples 13 and 18 (Table 1) showed a best fit with so far unidentified streptococcal clones according to GenBank entries.

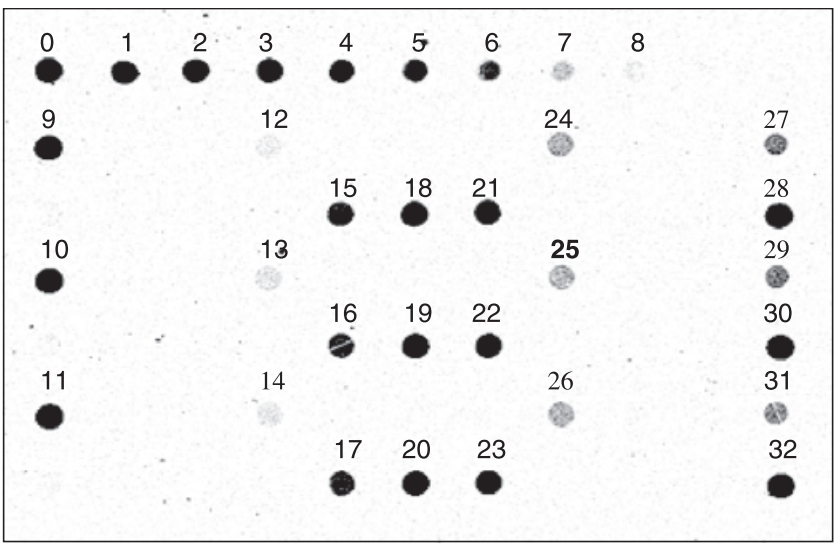

Fig. 2. Representative result (sample no. 17) of microarray analyses performed by the ParoCheck® DNA chip. Dot 0: orientation control. Dots 1–8: PCR control in 10-fold dilutions of control catcher probe. Dots 9–11 and 28, 30, 32: internal PCR positive controls. The sample was found to be positive in different intensities for Dots 12–14: *F. nucleatum;* Dots 15–17: *T. denticola;* Dots 18–20: *T. forsythia;* Dots 21–23: *P. gingivalis;* Dots 24–26: *V. parvula;* and Dots 27, 29, 31: *E. nodatum*. The sample was found to be negative for all the remaining 14 bacterial species mentioned in the text.

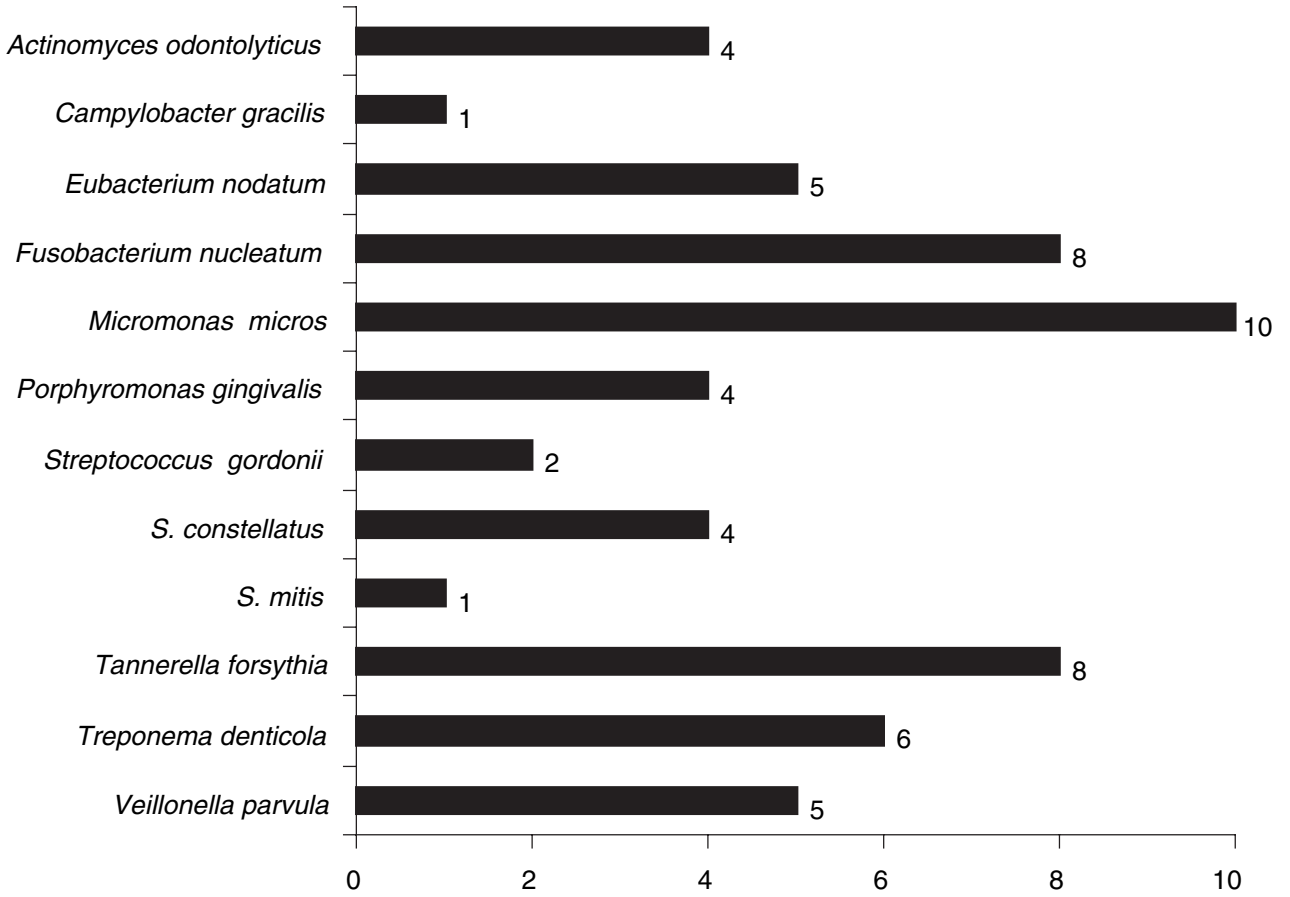

Fig. 3. Frequency of identifications from 20 necrotic root canals (microarray).

In one sample, no microorganisms were detected by culture methods alone. 'Sterile' endodontic samples have frequently been reported in culture-based studies (7–10, 12, 14, 23, 26, 28). The high number of facultative anaerobes found by culture in our study might be related to the absence of clinical symptoms (12).

Using microarrays, no microorganisms (out of the 20 included in the panel) were detected in three of the samples, none of which corresponded to the negative culture-based sample. Interestingly, the most prevalent organism detected by microarray, *M. micros*, found in 50% of the samples, was not recovered at all by culture analysis.

F. nucleatum was identified by both methods in four cases, and *P. gingivalis* as well as *S. mitis* in one case (Table 1). It is noteworthy that in a number of cases the culture-based approach failed to detect those bacteria identified by DNA-chip diagnostics and vice versa. For example, it was possible to identify *T. denticola* in 30% (6/20) of the cases by using the ParoCheck® assay but not by culturing (media for spirochetes were not used in this study). More remarkable is the prevalence of *T. forsythia* (40%, 8/20) according to DNA chip results, as this organism could be potentially recovered on the media used but is known to be very fastidious and oxygen-sensitive. These results are in agreement with the findings of Siqueira et al. (22) who examined the presence and levels of 42 bacterial species in 28 root canal samples by checkerboard DNA–DNA hybridization. Using this culture-independent technique they found *T. denticola* in 17.9% and *T. forsythia* in 39.3% of cases.

One reason for discrepancies between culture and DNA chip results could be the higher sensitivity of the PCR-based chip technology (detection limit 100–500 cells). Moreover, some media and conditions might favor the growth of facultative anaerobes, which then might inhibit or mask the colonies of obligate anaerobes, most likely explaining why *M. micros* was not recovered by our culture approach. Finally, commercial biochemical test systems used for species identification have their own limits, leading to the misclassification of isolates in some cases (6, 18).

However, DNA chips also have their limits. For instance, the DNA extraction procedure (lysis conditions) as well as the PCR-based techniques (access of primer to template sequence) might lead to biased retrieval of amplicons, discriminating against some bacterial species (16, 24). There are of course also the predefined limits on the range of organisms that can be detected by microarrays so far. As mentioned above, the microarray used in this study was originally developed to detect periodontopathogens, but all of the species covered have also been described in endodontic infections (19). However, some 'typical' endodontic species, such as *Porphyromonas endodontalis*, *E. faecalis*, *Pseudoramibacter alactolytis*, *Filifactor alocis*, or *Dialister pneumosintes* are not represented on the DNA-chip, but could be included in a future format adapted to situations with both perio- and endodontic infections.

Nonetheless, the principal effectiveness of the microarray-based identification of bacteria involved in endodontic infection, as reported here, demonstrates the potential value of this technique for monitoring

mixed-infection diseases. As the number of positions on a glass chip can reach hundreds of thousands, probes for well known pathogens and questionable or new bacterial species (i.e. only known from sequencing PCR clones and without culture pendant) [15, 17, 20, 21] and, finally, probes for the whole oral microflora (under physiological or pathological conditions) could be included.

In conclusion, to overcome the limitations of either method used alone, we have shown that the combined application of microarray technology and culture analysis is useful for increasing the range of detectable species in endodontic infections. This technology is useful for infectious processes where fastidious or obligate anaerobic bacteria are expected.

Acknowledgments

We thank Ilse Seyfarth, Rogerio C. Jacinto, Vreni Merriam, and Diane M. Citron for various forms of assistance. This work was supported by the Programme Alban, European Union Program of High Level Scholarships for Latin America, identification number E03D10234BR.

References

1. Brauner AW, Conrads G. Studies into the microbial spectrum of apical periodontitis. Int Endod J 1995: **28**: 244–248.
2. Bryant PA, Venter D, Robins-Browne R, Curtis N. Chips with everything: DNA microarrays in infectious diseases. Lancet Infect Dis 2004: **4**: 100–111.
3. Campbell CJ, Ghazal P. Molecular signatures for diagnosis of infection: application of microarray technology. J Appl Microbiol 2004: **96**: 18–23.
4. Chan VL. Bacterial genomes and infectious diseases. Pediatr Res 2003: **54**: 1–7.
5. Conrads G, Gharbia SE, Gulabivala K, Lampert F, Shah HN. The use of a 16S rDNA directed PCR for the detection of endodontopathogenic bacteria. J Endod 1997: **23**: 433–438.
6. Downes J, King A, Hardie J, Phillips I. Evaluation of the Rapid ID 32A system for identification of anaerobic Gram-negative bacilli, excluding the *Bacteroides fragilis* group. Clin Microbiol Infect 1999: **5**: 319–326.
7. Gomes BP, Lilley JD, Drucker DB. Associations of endodontic symptoms and signs with particular combinations of specific bacteria. Int Endod J 1996: **29**: 69–75.
8. Gomes BP, Lilley JD, Drucker DB. Clinical significance of dental root canal microflora. J Dent 1996: **24**: 47–55.
9. Gomes BP, Lilley JD, Drucker DB. Variations in the susceptibilities of components of the endodontic microflora to biomechanical procedures. Int Endod J 1996: **29**: 235–241.
10. Gomes BP, Pinheiro ET, Gade-Neto CR, et al. Microbiological examination of infected dental root canals. Oral Microbiol Immunol 2004: **19**: 71–76.
11. Guschin DY, Mobarry BK, Proudnikov D, Stahl DA, Rittmann BE, Mirzabekov AD. Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. Appl Environ Microbiol 1997: **63**: 2397–2402.
12. Jacinto RC, Gomes BP, Ferraz CC, Zaia AA, Filho FJ. Microbiological analysis of infected root canals from symptomatic and asymptomatic teeth with periapical periodontitis and the antimicrobial susceptibility of some isolated anaerobic bacteria. Oral Microbiol Immunol 2003: **18**: 285–292.
13. Kakehashi S, Stanley HR, Fitzgerald RJ. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg Oral Med Oral Pathol 1965: **20**: 340–349.
14. Molander A, Reit C, Dahlen G, Kvist T. Microbiological status of root-filled teeth with apical periodontitis. Int Endod J 1998: **31**: 1–7.
15. Munson MA, Pitt-Ford T, Chong B, Weightman A, Wade WG. Molecular and cultural analysis of the microflora associated with endodontic infections. J Dent Res 2002: **81**: 761–766.
16. Nadkarni MA, Martin FE, Jacques NA, Hunter N. Determination of bacterial load by real-time PCR using a broad-range (universal) probe and primers set. Microbiology 2002: **148**: 257–266.
17. Paster BJ, Boches SK, Galvin JL, et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
18. Santala AM, Sarkonen N, Hall V, Carlson P, Jousimies-Somer H, Kononen E. Evaluation of four commercial test systems for identification of *actinomyces* and some closely related species. J Clin Microbiol 2004: **42**: 418–420.
19. Siqueira JF Jr. Taxonomic changes of bacteria associated with endodontic infections. J Endod 2003: **29**: 619–623.
20. Siqueira JF Jr, Rocas IN. PCR methodology as a valuable tool for identification of endodontic pathogens. J Dent 2003: **31**: 333–339.
21. Siqueira JF Jr, Rocas IN, Rosado AS. Investigation of bacterial communities associated with asymptomatic and symptomatic endodontic infections by denaturing gradient gel electrophoresis fingerprinting approach. Oral Microbiol Immunol 2004: **19**: 363–370.
22. Siqueira JF Jr, Rocas IN, Souto R, de Uzeda M, Colombo AP. Checkerboard DNA-DNA hybridization analysis of endodontic infections. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2000: **89**: 744–748.
23. Sundqvist G, Figdor D, Persson S, Sjogren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 1998: **85**: 86–93.
24. Suzuki MT, Giovannoni SJ. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl Environ Microbiol 1996: **62**: 625–630.
25. Syed SA, Loesche WJ. Survival of human dental plaque flora in various transport media. Appl Microbiol 1972: **24**: 638–644.
26. Wasfy MO, McMahon KT, Minah GE, Falkler WA Jr. Microbiological evaluation of periapical infections in Egypt. Oral Microbiol Immunol 1992: **7**: 100–105.
27. Xiang CC, Chen Y. cDNA microarray technology and its applications. Biotechnol Adv 2000: **18**: 35–46.
28. Yoshida M, Fukushima H, Yamamoto K, Ogawa K, Toda T, Sagawa H. Correlation between clinical symptoms and microorganisms isolated from root canals of teeth with periapical pathosis. J Endod 1987: **13**: 24–28.