NEW MICROBIOLOGICA, **31**, 383-391, 2008

Comparison of culture methods and multiplex PCR for the detection of periodontopathogenic bacteria in biofilm associated with severe forms of periodontitis

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SUMMARY

Conventional culture methods and Multiplex PCR, both of which we have been used for a long time in our clinical microbiology laboratory, were compared for their ability to detect a selected panel of periodontopathic bacteria: *Aggregatibacter actinomycetemcomitans, Campylobacter rectus, Eikenella corrodens, Fusobacterium nucleatum, Porphyromonas gingivalis,* and *Prevotella intermedia.* Tests were performed in a single subgingival sample taken from a periodontal diseased site with a probing depth equal to or greater than 6mm. The results were compared site-by-site, taking into account the quality and the presence or absence of pathogens.

529 samples of subgingival plaque were analysed and the prevalence of the six species monitored varied in relation to the species itself and the method of detection. The most represented species is *F. nucleatum,* with a percentage of positive variability between 44.9% PCR and 46.5% culture test. Generally, the lowest prevalence was determined by culture test, with the exception of *E. corrodens* and *F. nucleatum,* which, unlike other bacteria, have been seen in higher percentages in culture with respect to PCR. For both methods, there was a good degree of accuracy in the determination of *A. actinomycetemcomitans*, *C. rectus*, *E. corrodens*, and *P. gingivalis*. It becomes weak for *F. nucleatum* and *P. intermedia*. Both culture and PCR techniques introduced many methodological problems when applied in oral microbiology, but the ideal technique for accurate detection of pathogens in subgingival plaque samples has yet to be developed.

KEY WORDS: Culture methods, Multiplex PCR, Sensitivity, Specificity, Accuracy

Received November 14, 2007 *Accepted* February 20, 2008

INTRODUCTION

The microbial populations involved in periodontal diseases are known to be highly complex and variable and have not yet been fully identified, although key organisms are generally recognised to be associated with disease progression.

Strong positive associations and the concurrent presence of the bacterial species *Campylobacter rectus, Prevotella intermedia, Porphyromonas gingivalis*, and *Tannerella forsythensis* were described in adult periodontitis (Kamma *et al.,* 1999). In addition, other bacteria such as *Aggregatibacter actinomycetemcomitans, Eikenella corrodens, Fusobacterium nucleatum,* and *Treponema denticola* are considered putative periodontopathogenic microorganisms (Haffajee *et al.,* 2006).

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Bacterial culturing has been the classic diagnostic method widely used in the study of the composition of dental plaque and is still generally used as the gold or primary standard when determining the utility of a new microbial test in periodontal microbiology (Lau *et al.,* 2004; Verner *et al.,* 2006).
The main advantages of this method are its capacity to detect multiple bacterial species simultaneously and the possibility of obtaining relative and absolute counts of cultured species. Moreover, it is the only method that can detect unexpected bacteria, characterise new species and permit the assessment of antibiotic sensitivity of the grown bacteria (Loomer 2004; Sanz *et al.,* 2004; Socransky *et al.,* 1998; Eick *et al.,* 2002). In contrast to the other detection methods, positive findings based on cultivation can be confirmed by subsequent testing.
However, culture techniques have many methodological problems when used in oral microbiology and attempts to culture anaerobic bacteria from periodontal pockets result in a significant underestimation of the quantity of *in situ* bacteria. Cultural procedures rely on the detection of viable organisms and require samples to be immediately processed upon acquisition in order to maximize bacterial survival, in conjunction with essential strict transport conditions. This method demonstrated an inability or difficulty in growing several bacterial species, e.g. *T. forsythensis* is uncultivable or extremely difficult to cultivate, spirochetes can account for 40% of the microscopic count but they are not grown by cultural procedures (Loesche *et al.,* 1992).
In addition, the anaerobic procedures themselves have many method error sources and only presumably identify putative pathogens, because the additional tests can sometimes be inconclusive. Additional errors can be associated with the sampling procedure, the media used, the degree of anaerobiosis employed and the type of dispersal procedures used. The magnitude of these errors may vary with each of the cultivable species found in plaque and could be as high as fivefold with some of the more fastidious species (Jervoe-Storm *et al.,* 2005).
Finally, the cultures require specific laboratory equipment and experienced personnel, are labour intensive, expensive, time-consuming and need a prolonged period before results can be obtained. Such technical difficulties with culture methods suggested the need for a specific and rapid technology that does not require the preservation of viable microorganisms, and which could be a more rapid method of identifying multiple bacterial species coincidentally. This need has led to the development of other bacterial detection methods, including the PCR.
The Polymerase Chain Reaction (PCR) fulfils this need since it can specifically identify microorganisms in clinical samples by testing for the presence of species-specific sequences of DNA. It is extremely sensitive, being able to detect even one copy of the searched DNA target and does not require rigorous conditions for transport of samples from the clinical department to the laboratory (Sanz *et al.,* 2004).
Riggio *et al.* (1996) reported that PCR is a powerful diagnostic tool that can detect low numbers of periodontal pathogens with a high degree of accuracy in subgingival plaque samples. It is rapid, with results being available within hours of sample acquisition, cheaper and less labour-intensive than conventional culture methods and permits many more samples to be easily screened at one time. With these advantages in mind, they proposed that PCR should be regarded as the "gold standard" for identifying major periodontal pathogens in subgingival plaque samples.
The purpose of this study was to compare the ability of culture methods and Multiplex PCR to detect a selected panel of periodontopathic bacteria in a single subgingival sample taken from a periodontal diseased site with a probing depth equal to or greater than 6mm.
Results of the comparison would serve to determine which procedure would be best to use as the reference procedure.

MATERIAL AND METHODS

Patients and samples

A total of 529 subgingival plaque samples were collected from patients with advanced chronic periodontitis (mean age 63±10.4 years; range 41-77 years) with probing depth (PD) values equal or more than 6 mm in the experimental sites. The subjects had to comply with the following criteria:

1. positive for diagnosis of mild-to-severe chronic periodontitis;

2. good general health in accordance with their medical history, blood pressure, pulse rate;
3. negative for the use of any antibiotic or anti-inflammatory drugs within the 6 months preceding the beginning of the study.

Subgingival plaque was collected for microbiological evaluation at the predetermined site during a screening visit as follows: the sites were isolated with cotton rolls; removal of supragingival plaque by a sterile curette (Asadental, Bozzano, Italy); gingival surface was dried and plaque samples were obtained by insertion of three standardised #30 sterile paper points (Inline, Turin, Italy) at the deepest part of each periodontal pocket and left *in situ* for 15 s. The paper points were transferred to a test tube containing 1 ml of the VMGA III transport medium under anaerobic conditions, and immediately sent to the microbiological laboratory. After thoroughly shaking by vortex for 60 s, 500 µl of the transport medium was used for culturing method, and 500 µl for PCR analysis.

Microbiological procedures

Culture conditions

Generally, isolation of microorganisms was carried out by methods previously reported (Paolantonio *et al.,* 2004). In particular, in the case of some bacterial strains, special microbiological procedures were applied. Each sample was subjected to a series of 10-fold dilutions (to 10^{-4}) in 0.1 M phosphate buffer and aliquots of 100 µl from each dilution were spread onto different selective media.

In brief, the following plates were inoculated and incubated at 37°C for 7 days in an anaerobic chamber (80/10/10, $N_2/H_2/CO_2$; Don Whitley Scientific Ltd, Shipley, UK; International PBI SpA): CVE (trypticase soy agar, yeast extract 5 g/l, sodium chloride 5 g/l, glucose 2 g/l, tryptophan 0.2 g/l, crystal violet 5 mg/l, erythromycin 4 mg/l, defibrinated sheep blood) (Walker *et al.,* 1979), to assess *Fusobacterium nucleatum*; KVLB-2 (kanamycin 75 ug/ml, vancomycin 2 ug/ml, laked blood) to assess the black-pigmented *Porphyromonas gingivalis* and *Prevotella intermedia* (Kamma *et al.,* 1999); Wolinella agar (trypticase soy agar, vancomycin 9 ug/ml, ferrous sulphate 0.2 g/l, sodium thiosulphate 0.3 g/l, sodium fumarate 3 g/l, sodium formate 2 g/l) for the isolation of *Campylobacter rectus* (*Kamma et al.,* 1999), Brucella agar (BA) plates enriched with 5% defibrinated sheep blood and 5 ug/ml clindamycin to assess *Eikenella corrodens* (Goldstein *et al.,* 1983). A definitive identification of all representative isolates was then obtained by subculturing onto Brucella blood agar (Oxoid) followed by inoculation of purified cultures onto a commercially packaged automated system (BioMérieux Italia SpA, Rome, Italy).

Aliquots of 0.1 ml of the appropriate dilution were also spread onto TSBV (trypticase soy, serum, bacitracin 75 ug/ml, vancomycin 5 ug/ml) agar plates, a selective medium for *Aggregatibacter actinomycetemcomitans* (Slots 1982) and then cultured at 37°C in a microaerophilic environment (5% CO_2, 95% N_2). After 7 days, the TSBV agar plates were examined for the presence of *A. actinomycetemcomitans*. Small translucent, slightly convex, circular colonies, often with a star-like inner structure and adherent to the agar surface, were subcultured for identification. A definitive identification was made on the basis of Gram stain, nitrate reduction, production of catalase, urease and indole, growth on McConkey agar and fermentation reactions to carbohydrates (fructose, glucose, lactose, maltose, mannitol, sucrose and xylose) supplemented by the profiles of preformed enzymes (API-ZIM System, BioMérieux Italia SpA, Rome, Italy). For each microbial species, data were recorded as the count of CFUs/ml on the growth plate.

DNA-extraction

Nucleic acids were extracted within 24 to 48 hours from specimen collection. The samples were vortex-mixed and centrifuged to collect the cells. The pellet was suspended in 300 µl of lyses buffer (50 mM Tris, 10 mM EDTA and 10% SDS) plus lysozyme (5 mg/ml) and incubated at 37°C for 1 h. Then, 125 µg of proteinase-K was added and after 1 hour incubation at 65°C, the DNA was extracted with phenol and chloroform-isoamyl alcohol treatment. Nucleic acids were precipitated in alcohol, washed with 70% (vol/vol) alcohol and suspended once more in sterile water. The DNA extracted from each sample was assayed by multiplex PCR for the detection of *A. actinomycetemcomitans, C. rectus, E. corrodens, F. nucleatum, P. gingivalis, and P. intermedia.*

PCR-detection

Multiplex PCR was performed using specific primers for the 16S rRNA gene of each bacterium

(Santangelo *et al.,* 2004; D'Ercole *et al.,* 2006). PCR amplification reactions were carried out in a reaction mixture in a final volume of 100 µl consisting of 10 µl of DNA sample, and 90 µl of reaction mixture containing 30 pmol of each primer, 200 µM of a mixture of deoxynucleoside triphosphates, 1.5 mM MgCl2, 1 x PCR buffer (10 mM Tris-HCl, pH 8.0), 50 mM KCl, 2.5 U Hot Start Taq™ DNA Polymerase (Quiagen S.p.a., Milan, Italy). The PCR protocol was as follows: 98°C for 15 min followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a final step of 72°C for 10 min.
PCR amplification was performed in an iCycler System (BioRad Laboratories Srl, Segrate, Milan, Italy). Amplicons were detected by electrophoresis of 20 µl of samples from each PCR tube in a 2% agarose gel in TAE (Tris-Acetate-EDTA buffer) for 2 h at 80 V. The amplification products were visualized and photographed under a UV light trans illuminator (Gel Doc 2000, BioRad) after 30 min of ethidium bromide (1 µg/ml) staining. A positive or negative identification was based on the presence of clear bands of the expected molecular size using a commercial DNA molecular weight marker (number VIII; Roche Diagnostics S.p.A., Milan, Italy). Each assay was performed once, and in case of disagreeing results, the assay was repeated once more.

Data analysis

The results were compared site-by-site, taking into account the quality and the presence or absence of pathogens. For the analyses, the ordinal responses were dichotomized, with a value of zero indicating the absence and a value of one indicating the presence of bacteria. Empirical estimators of sensitivity, specificity, positive and negative likelihood ratios, positive and negative predictive value, observed and expected accuracy were performed, using culture as the diagnostic reference standard. This analysis was repeated using the PCR results as the reference standard. The level of detection of the presence of bacteria for the various analyses was as follows: anaerobic culture $\geq 10^3$-10^4 CFU, multiplex PCR $\geq 10^2$-10^3 CFU.
For measuring the agreement between the anaerobic cultivation and multiplex PCR kappa-statistics (K) was used. For the description of the agreement of the results of both methods, the Spearman correlation coefficient (SPSS software package, version 11.0) was calculated.

RESULTS

The frequency of detection of the six periodontopathogens as identified by culture and multiplex PCR are reported in Table 1.
An important relationship between the presence of periodontopathogenic bacteria and the serious form of periodontal disease was analysed. In particular, in the 529 samples of subgingival plaque analysed, the prevalence of the 6 species monitored varied as regards the species itself and the method of detection.
The most represented species is *F. nucleatum,* with a percentage of positive variability between 44.9% PCR and 46.5% culture test. The difference between culture method and PCR was pointed out when determining all the species analysed. Generally, the lowest prevalence was determined with the culture test, with the exception of *E. corrodens* and *F. nucleatum,* which contrary to other bacteria were seen in higher percentages of culture with respect to PCR.
The results, in relation to the two techniques, using both culture and PCR as reference assay, are

TABLE 1 - *Presence of selected periodontopathic bacteria determined by two separate detection methodologies in subgingival plaques taken from diseased sites.*

	Detection methodology	
	Culture +	*PCR* +
Aggregatibacter actinomycetemcomitans	10.77	24.57
Campylobacter rectus	25.52	42.34
Eikenella corrodens	*45.18*	*32.7*
Fusobacterium nucleatum	46.5	44.99
Porphyromonas gingivalis	10.96	17.77
Prevotella intermedia	22.87	31.76

Percentage of sites in which the indicated species was present in the plaque

reported in the Tables 2-7. The statistic analysis disclosed low and conflicting values in terms of sensitivity, specificity, positive and negative likelihood ratios, positive and negative predictive value, observed accuracy, expected accuracy of both the analysed techniques compared to each other. The degree of accuracy among cultures and PCR was rather low.

It is possible to emphasize a coefficient of accuracy (k) of the value of 0.21 in the determination

TABLE 2 - *Comparison between culture and PCR procedures in their ability to detect Aggregatibacter actinomycetemcomitans in subgingival plaques.*

	Reference procedure														
Test procedure	*Culture*						*PCR*								
	SE	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*SE*	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*AO*	*AA*	*K*
Culture							23.84	93.48	0.36	0.81	54.38	79.02	76.37	70	0.21
PCR	54.39	79.03	2.6	0.58	23.85	93.48									

SE = Sensitivity %; SP = Specificity %; LR+ = positive likelihood ratio; LR-= negative likelihood ratio; VPP = positive predictive value; VPN = negative predictive value; AO = observed accuracy%; AA = expected accuracy; K = agreement between two methods

TABLE 3 - *Comparison between culture and PCR procedures in their ability to detect Eikenella corrodens in sub gingival plaques.*

	Reference procedure														
Test procedure	*Culture*						*PCR*								
	SE	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*SE*	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*AO*	*AA*	*K*
Culture							65.89	64.88	1.87	0.52	47.69	79.65	65.22	51.67	0.28
PCR	47.7	79.66	2.3	0.7	65.9	64.9									

SE = Sensitivity %; SP = Specificity %; LR+ = positive likelihood ratio; LR-= negative likelihood ratio; VPP = positive predictive value; VPN = negative predictive value; AO = observed accuracy%; AA = expected accuracy; K = agreement between two methods

TABLE 4 - *Comparison between culture and PCR procedures in their ability to detect Campylobacter rectus in sub gingival plaques.*

	Reference procedure														
Test procedure	*Culture*						*PCR*								
	SE	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*SE*	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*AO*	*AA*	*K*
Culture							37.05	82.95	2.7	0.75 63.52	61.48 53.75	64.21 0.21			
PCR	61.48	64.21	1.71	0.59	37	82.9									

SE = Sensitivity %; SP = Specificity %; LR+ = positive likelihood ratio; LR-= negative likelihood ratio; VPP = positive predictive value; VPN = negative predictive value; AO = observed accuracy%; AA = expected accuracy; K = agreement between two methods

of *A. actinomycetemcomitans*, *C. rectus*, of 0.28 for *E. corrodens*, and of 0.36 for *P. gingivalis*, which is considered as a good degree of accuracy. It becomes weak for *F. nucleatum* (0.20) and *P. intermedia* (0.16).

DISCUSSION

This study compared a molecular method, PCR, for highly preserved regions 16S rDNAs and the conventional culture method used for a long time

TABLE 5 - *Comparison between culture and PCR procedures in their ability to detect Fusobacterium nucleatum in subgingival plaques.*

	Reference procedure														
Test procedure	*Culture*						*PCR*								
	SE	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*SE*	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*AO*	*AA*	*K*
Culture							57.56	62.54	1.53	0.67	55.69	64.31	60.3	50.35	0.20
PCR	55.69	64.31	1.56	0.68	57.56	62.54									

SE = Sensitivity %; SP = Specificity %; LR+ = positive likelihood ratio; LR-= negative likelihood ratio; VPP = positive predictive value; VPN = negative predictive value; AO = observed accuracy%; AA = expected accuracy; K = agreement between two methods

TABLE 6 - *Comparison between culture and PCR procedures in their ability to detect Porphyromonas gingivalis in subgingival plaques.*

	Reference procedure														
Test procedure	*Culture*						*PCR*								
	SE	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*SE*	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*AO*	*AA*	*K*
Culture							36.17	94.48	6.55	0.67	58.62	87.26	84.12	75.16	0.36
PCR	87.26	58.62	4.6	0.47	36.17	94.48									

SE = Sensitivity %; SP = Specificity %; LR+ = positive likelihood ratio; LR-= negative likelihood ratio; VPP = positive predictive value; VPN = negative predictive value; AO = observed accuracy%; AA = expected accuracy; K = agreement between two methods

TABLE 7 - *Comparison between culture and PCR procedures in their ability to detect Prevotella intermedia in subgingival plaques.*

	Reference procedure														
Test procedure	*Culture*						*PCR*								
	SE	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*SE*	*SP*	*LR+*	*LR-*	*VPP*	*VPN*	*AO*	*AA*	*K*
Culture							33.33	81.99	1.85	0.81	46.28	72.54	66.54	59.89	0.16
PCR	46.28	72.55	1.68	0.74	33	81.99									

SE = Sensitivity %; SP = Specificity %; LR+ = positive likelihood ratio; LR-= negative likelihood ratio; VPP = positive predictive value; VPN = negative predictive value; AO = observed accuracy%; AA = expected accuracy; K = agreement between two methods

in our oral microbiology laboratory, in determining the principal pathogens in the samples of subgingival plaque of patients with periodontal diseases. These two techniques were compared because their detection limit was similar (10^3-10^4 cells for anaerobic cultures, 10^2-10^3 cells for multiplex PCR).

At the beginning, as a point of comparison for our multiplex PCR, the culture examination was chosen because it has long been considered the gold standard. The new technique shows a low sensitivity with higher values of specificity, with scarce possibilities therefore of false positives in the search for *A. actinomycetemcomitans*, *E. corrodens* and *P. intermedia*. On the other hand, it has the best sensitivity and poor specificity in the search of *P. gingivalis*.

This study also calculated the diagnostic validity of culture using PCR as the standard reference, as suggested by Riggio *et al.* (1996). The culture method shows a high precision in the possibility of identifying false positives (high specificity), superior to that shown in the contrary hypothesis, in the determination of *A. actinomycetemcomitans*, *C. rectus, P. gingivalis*, and *P. intermedia*. In the absence of a standard of reference, each technique has the same probability of demonstrating the truth. To determine the degree of accuracy between the two methods, they were analyzed according to the perspective that both methods are procedures of reference and test procedures of the examined microorganisms. This can be considered a good strategy, which however observes the problem of evaluation of the characteristics of a diagnostic test in the absence of a gold standard (a very common situation, considering that the presence of a standard is an exception).

The traditional standard used in parodontal microbiology, dilutions in series of anaerobic crops, appears to be the more deficient research method compared to PCR, having made the lowest values of prevalence for four of the six examined microorganisms.

E. corrodens and *F. nucleatum* are the prevailing species, being present in 45.18% (culture) and 46.5% (PCR) of the samples respectively. *P. gingivalis* is the species least represented since it was positive to the culture examination only in 10.96% of cases. The study confirmed the strong association of analyzed bacteria with severe forms of periodontitis and the results obtained for *A. actinomycetemcomitans P. gingivalis E. corrodens* and *F. nucleatum* are in accordance with those of previous studies. (Riggio *et al.,* 1996; Mullaly *et al*., 2000; Kamma *et al.,* 2004; Ledder *et al.,* 2007). For *P. intermedia* and *C. rectus* our study noted an inferior prevalence to those indicated by Mullaly *et al*. (2000) and Kamma *et al.* (2004), but also in the range of different studies (Sanz *et al.,* 2004). The explanation can be looked for in terms of selected patients, methods used and geographical differences (Herrera *et al.,* 2008).

In the search for *F. nucleatum* and *E. corrodens*, the culture method furnishes a greater percentage in comparison to PCR. There are numerous matters that explain the variations of the levels of determination reached by the two methods. The most probable theory is related to the fact that the culture medium is not specific for *F. nucleatum* and *Fusobacterium periodonticum*. The analysis can thus induce an overestimate by confusion of colonies, which is not the case for PCR. The low sensitivity shown by PCR in comparison to culture, in the examination of *E. corrodens* and *F. nucleatum* is to be considered due either to the extreme competitiveness that characterizes the subgingival flora, or to the incorrect biochemical identification of the colonies or to the scarce specificity of the primers, or to the technical inadequacies or to a change of bases in the gene among the different strains that can prevent bonding of the primers to their sequence target. The possibility that in the PCR the primers can hybrid with DNA of near or predominant species was minimized through accurate selection of the primers and tests of cross-reaction, but likewise, false positives or negatives can be caused by non specific imputable bonds to the same technique or to a certain genetic variability.

However, it is more difficult to justify the culture positive PCR negative. This case could mean a false positive identified by culture. This has been reported with the species *Haemophilus aphrophilus* which can show cross-reactivity with some of the biochemical tests used to identify *A. actinomycetemcomitans* (Lau *et al.,* 2004). Another possibility is the presence of false negatives with PCR. This could be justified by the presence of the genetic variations in their sequence.

Once verified that the prevalence of an organism in the sample of subgingival plaque is functioning

through the method of observation and of the microorganism itself, it can be claimed that in reality none of the two methods can be considered the standard of reference.

From the analysis of our results and from those in literature it is possible to observe that no one wants to block the use of the culture method for the search of oral microorganisms, still valid today as shown by our work (see example of *E. corrodens, F. nucleatum*) but it is suggested that the new molecular method can determine these microorganisms more accurately.

Despite the passing of the years and the large number of studies, much confusion persists on which method is more appropriate for the search of periodontopathogenic bacteria, considering also that recent works by different authors continue to consider the great utility that the culture method continues to offer despite the advent of real time PCR and more sensitive DNA probes (Lau *et al.,* 2004; Verner *et al.,* 2006).

Culture methods have the advantage of being able to detect a wide variety of species; the characterization of all isolates may allow the identification of unexpected or new species. This technique enables us to search for all the microorganisms present in a non-specific way and it remains the most objective technique (gold standard). However, taxa present in low proportions might be missed unless selective culture techniques are used, but such techniques are often too suppressive.

PCR can by-pass many of the restrictions of anaerobic cultures and have a lower detection limit than non-selective cultures, but they are only applicable to pre-selected target species for which antibodies of known specificity must be available (Teoman *et al.,* 2007). One significant advantage of this multiplex PCR is the multitarget analysis, i.e. it can detect more than one bacterial species at a time.

The choice could depend on the demand in time for the analysis. PCR can provide results in 2h, whereas anaerobic cultures require 7-8 days to confirm the presence of putative periodontopathogens (and nearly an extra week for the antibiogram dates).

Although interest in the culture technique remains relative, because of the presence of biofilm and the complexity of the oral flora, we still have no other tested scientific method by which to analyze the sensitivities of pathogens to antibiotics (D'Ercole *et al*., 2008).

In conclusion, the ideal technique for accurate detection of pathogens in subgingival plaque samples has yet to be developed. The high sensitivity and specificity of multiplex PCR justifies its use in epidemiological studies of periodontal diseases. Both these techniques can detect multiple bacterial species coincidentally, but the bacterial cultures can detect unexpected bacteria and also allow the determination of antibiotic resistance.

ACKNOWLEDGMENTS

The Authors are grateful to Ray Pizzuto (M.A. English Language, Oxford University) for reading the text.

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