Additive or Synergistic Antimicrobial Effects of Amoxicillin and Metronidazole on Whole Plaque Samples: A Preliminary Report

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Abstract

Objective: *In vitro* data on the susceptibility of oral bacteria to the combination of metronidazole and amoxicillin is limited. The aim of this preliminary study was to determine the susceptibility of whole subgingival plaque samples to amoxicillin and metronidazole and to their combination. **Methods:** Prior to any treatment procedures subgingival plaque samples from patients with severe generalized periodontitis were taken. Appropriate dilutions were plated on Columbia blood agar supplemented with the following agents: 3 µg/mL amoxicillin, 8 µg/mL amoxicillin, 8 µg/mL metronidazole, 16 µg/mL metronidazole, 3 µg/mL amoxicillin plus 8 µg/mL metronidazole or 8 µg/mL amoxicillin plus 16 µg/mL metronidazole. All plates were incubated anaerobically at 36°C for 14 days and the colony forming units (CFU) were determined. **Results:** Both applied metronidazole concentrations were able to decrease the CFU counts by approximately one order of magnitude in a log10 scale. Amoxicillin 3 µg/mL revealed a reduction of 2.4 log10 CFU, whereas 50% of the samples did not grow on the plates supplemented with 8 µg/mL of amoxicillin. There was no anaerobic bacterial growth on agar plates supplemented with the combination of amoxicillin and metronidazole even at the lower antibiotic concentrations. **Conclusion:** Susceptibility screening of subgingival samples to metronidazole and amoxicillin and to their combination seems to offer a rational basis for the selection of adjunctive antibiotic therapy

Key words: Antibiotics, synergistic effect, aggressive periodontitis, metronidazole, amoxicillin

Introduction

Periodontal diseases are multifactorial biofilm-associated infections. A distinct differentiation between aggressive and chronic forms is difficult (Meyer *et al.*, 2004), even on the basis of microbiological findings (Mombelli *et al.*, 2002; Ximenez-Fyvie *et al.*, 2006; Schacher *et al.*, 2007). Hence, the diagnosis of "aggressive periodontitis" is primarily based on clinical and radiological characteristics, on patient's age, and on findings derived during clinical follow-up. Due to the infection-induced nature of periodontal diseases, antimicrobial therapies based on microbiological examinations may improve the treat-ment outcome of advanced and/or aggressive forms of periodontitis.

First attempts to control periodontal diseases with the adjunctive use of antibiotics included systemic administration of tetracyclines, amoxicillin with or without clavulanic acid, clindamycin and metronidazole (Listgarten *et al.*, 1978; Lekovic *et al.*, 1983; Gordon *et al.*, 1985; Magnusson *et al.*, 1989). Another adjunctive treatment approach was topical administration of various antibiotics or antiseptics (Lindhe *et al.*, 1979; Needleman and Watts, 1989; Stabholz *et al.*, 2000). Two decades ago, the combination of metronidazole and amoxicillin - so called "van Winkelhoff-Cocktail" - was introduced as an adjunctive systemic therapy for periodontitis treatment (Van Winkelhoff *et al.*, 1989). This regimen was specifically designed for treatment of diseases associated with *Aggregatibacter (Actinobaccillus) actinomycetemcomitans*, for which a synergistic *in vitro* effect between the two substances or their metabolites has been reported

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(Pavicic *et al.*, 1994a; Pavicic *et al.*, 1994b). Clinical studies on aggressive forms of periodontitis have revealed improved outcomes within observation periods up to five years, provided that the adjunctive treatment with the combination of amoxicillin and metronidazole was strictly combined with mechanical biofilm removal (Buchmann *et al.*, 2002; Guerrero *et al.*, 2005; Kaner *et al.*, 2007a; Kaner *et al.*, 2007b). Moreover, improved clinical advantages of this regimen were found in a placebo-controlled study comparing the antibiotic combination to the agents alone, again as adjunctive to mechanical, non-surgical periodontal treatment (Rooney *et al.*, 2002). In this report, the treatment outcomes of subjects with advanced chronic periodontal disease were independent from the initial microbiological findings. Recently, this strategy of combining amoxicillin and metronidazole was used for the treatment of generalized "aggressive periodontitis" without targeting against specific microorganisms (Guerrero *et al.*, 2005).

Whenever antibiotics are administered as an adjunctive periodontal treatment, existing or possibly developing resistance of the associated microflora should be carefully considered. *In vitro* findings have suggested that there are remarkable differences in resistance profiles of certain oral bacterial species (Van Winkelhoff *et al.*, 2005; Lakhssassi *et al.*, 2005). Recent findings in microbiological susceptibility testing have indicated the rationale of the examination of mixed microbial cultures instead of, or in addition to, the individual disease-associated strains (Karbach *et al.*, 2007). Such *in vitro* data about bacterial susceptibility to the combination of amoxicillin and metronidazole is hitherto scarce.

The aim of the present preliminary study was to determine the susceptibility of whole subgingival plaque samples to amoxicillin and metronidazole and to their combination.

Materials and methods
Patients and sampling

Four generally healthy patients with severe generalized chronic or aggressive periodontitis were recruited from the pool of patients from the Department of Periodontology, Endodontology and Cariology at the School of Dental Medicine, University of Basel, Switzerland. Diagnosis was based on clinical and radiographic findings, related to age and the severity of destruction (*Table 1, Figure 1a-b,* Armitage, 1999). Clinical measurements of probing pocket depth and attachment level were performed with the probe PCPUNC-15 (Hu-Friedy, Chicago, IL, USA). All recruited patients (one female and three males with a mean age of 40.8 years) were current or former heavy smokers and had neither received any earlier periodontal treatment nor systemic or topical antibiotics one year prior to the sampling. The female patient was not pregnant.

Subgingival plaque samples were taken for antibiotic resistance analysis. At least the two deepest periodontal pockets with bleeding on probing were selected for microbiological sampling. Supragingival plaque was removed, the sampling site was isolated using cotton rolls and gently dried with air. A sterile paper point was inserted to the bottom of the pocket, left in place for 20 s and placed in 0.5 ml of thioglycolate broth (bioMérieux, Genf, Switzerland; Casas *et al.*, 2007).

Microbiological procedures

Immediately after sampling, pooled paper points were vortexed for one minute and serially diluted in thioglycolate broth. For the determination of the total anaerobic bacterial count, 100 mL of the dilutions were plated on Columbia blood agar plates (Columbia Agar Base [BBL Becton Dickinson, Allschwil, Switzerland] enriched with 4 mg/L hemin, 1 mg/L menadione, and 50 ml/L human blood).

For quantification of the proportion of microorganisms resistant to either amoxicillin and/or metronidazole, Columbia blood agar plates supplemented with the following concentrations of the respective antimicrobial agent were used: 3 mg/mL amoxicillin (Fluka, Buchs, Switzerland), 8 mg/mL amoxicillin, 8 mg/mL metronidazole (Fluka), 16 mg/mL metronidazole, 3 mg/mL amoxicillin plus 8 mg/mL metronidazole or 8 mg/mL amoxicillin plus 16 mg/mL metronidazole. The concentrations of the antibiotics were adopted from van Winkelhoff *et al.* (2000) and/or the Clinical Laboratory and Standards Institute (2007). All plates were incubated anaerobically (10% CO_2, 10% H_2, 80% N_2) at 36°C for 14 days and the colony forming units (CFUs) were determined.

Results
Microbial findings

Microbiological data are presented in *Table 2*. The total anaerobic plaque count (CFU) ranged from 3.1 x 10^6 to 7.2 x 10^7 among the plaque samples, and the percentage of black-pigmented bacteria ranged from 40 to 80%. All samples showed a decrease of bacterial growth on agar by approximately 1 log with both concentrations of the antibiotic agent (8 mg/mL and 16 mg/mL). All agar plates supplemented with 3 mg/mL amoxicillin showed a reduced bacterial growth by log 2.4, whereas two out of four samples revealed no growth on the plates supplemented with 8 mg/mL of amoxicillin (*Table 2*). On agar plates supplemented with the combination of amoxicillin and metronidazole, no anaerobic bacterial growth was detected even at lower antibiotic concentrations.

Table 1. Profile of study patients and clinical characteristics.

Patient	Age	Periodontal diagnosis	Smoking status	Number of teeth	Number of sites with PPD ≥ 6 mm	Number of sites with BOP+
1	45	GAgP	Former smoker 30 pack years	29	165	174
2	32	GAgP	Current smoker 15 pack years	29	92	133
3	38	GAgP	Current smoker 17 pack years	25	69	96
4	48	GChP	Current smoker 30 pack years	27	25	46

BOP, bleeding on probing; GAgP, generalized aggressive periodontitis; GChP, generalized chronic periodontitis

Figure 1a.
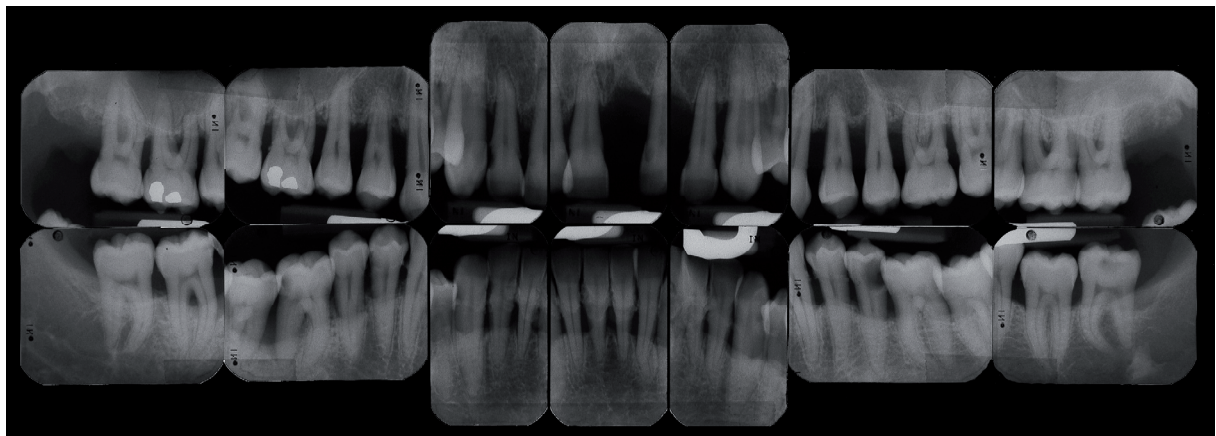
Figure 1b.

Figure 1. Patient N° 2 was diagnosed with generalized aggressive periodontitis due to extensive bone loss at the age of 32 years. a) Clinical intraoral photographs; b) Full-mouth periapical radiographs

Table 2. Microbiological characteristics and results of the antibiotic susceptibility analyses.

Patient	Bacterial growth on agar plates (control)		Bacterial growth on agar plates supplemented with different concentrations of metronidazole or amoxicillin					
	CFU	% BPB	CFU amoxicillin 3 µg/mL	CFU amoxicillin 8 µg/mL	CFU metronida-zole 8 µg/mL	CFU metronida-zole 16 µg/mL	CFU amoxicillin 3 µg/mL +metronidazole 8 µg/mL	CFU amoxicillin 8 µg/mL + metronidazole 16 µg/mL
1	4.0×10^7	40	9.0×10^4	-	6.0×10^6	1.5×10^7	-	-
2	1.0×10^7	50	8.0×10^3	1.0×10^4	6.0×10^5	6.0×10^5	-	-
3	3.1×10^6	40	8.4×10^4	-	6.0×10^5	1.3×10^6	-	-
4	7.2×10^7	80	4.0×10^5	3.0×10^5	1.3×10^6	1.5×10^6	-	-

BPB, black-pigmented bacteria; CFU, colony forming units

Discussion

The present preliminary study using subgingival plaque samples demonstrated reduced bacterial growth in the presence of low concentrations of metronidazole or amoxicillin, while higher amoxicillin concentrations inhibited bacterial growth in two out of four samples. Interestingly, the combination of metronidazole and amoxicillin was effective against microorganisms in all subgingival plaque samples at lower antibiotic concentrations. This *in vitro* observation suggests an additive or synergistic mode of action for these agents, which is likely to be beneficial for infection control, as demonstrated by recent clinical studies (van Winkelhoff *et al.*, 1989; Buchmann *et al.*, 2002; Rooney *et al.*, 2002; Guerrero *et al.*, 2005; Kaner *et al.*, 2007a; Kaner *et al.*, 2007b). It may be hypothesized that the targeted use of this additive/synergistic effect, which is either based on growth inhibition or on bacteriocidal effects, may offer a strategy against the development and/or the control of resistant strains.

The introduced method testing microbial susceptibility to a frequently administrated combination of antibiotics is a novel approach, which enlightens the capacity of additive and/or synergistic effects between the two substances. A synergistic effect of two antibiotics needs to be evaluated on a species level, and was documented for *Aggregatibacter actinomycetemcomitans* (Pavicic *et al.*, 1994a; Pavicic *et al.*, 1994b). The authors suggested a higher rate of metronidazole uptake by bacterial cells simultaneously incubated with amoxicillin. Resistance of anaerobic bacteria to metronidazole hardly ever occurred (Seifert and Dalhoff, 2010). In the current material, bacterial growth was detected in all four subgingival plaque samples, which is indicative of metronidazole-resistant strains and emphasizes the need for susceptibility testing in selected patients with infections involving anaerobic bacteria. The results of the current study should be, however, interpreted with caution due to the limited number of subjects included, and the lack of specific bacterial strain characterisation. However, the mixed subgingival plaque samples used here represented the expected general characteristics in terms of relative proportions of back-pigmented anaerobes in the total culturable flora.

This preliminary study was restricted to current or former heavy smokers, who have an increased risk for the onset and progression of periodontal diseases (Warnakulasuriya *et al.*, 2010). Cigarette smoking is likely to affect the composition of the oral microflora due to a decrease in oxygen tension in periodontal pockets, and may promotes a selection of anaerobic bacteria (Hanioka *et al.*, 2000). However, the literature has been indecisive as to whether a specific smoking-associated microbial profile exists (van Winkelhoff *et al.*, 2001; van der Velden *et al.,* 2003). Interestingly, recent evidence

from a randomized controlled trial suggests a benefit of adjunctive antimicrobial therapy with metronidazole and amoxicillin in the non-surgical periodontal treatment of smokers with chronic periodontitis (Matarazzo *et al.*, 2008).

The culture technique used in the current investigation may have some shortcomings: (i) restricted to growth of viable bacteria, (ii) strict sampling and transport conditions essential, (iii) specific laboratory equipment and experienced personnel required for bacterial culturing, (iv) time needed for bacterial growth on appropriate media, (v) specific pathogens in the subgingival plaque may not be detected. However, the main advantage of the technique used is the probability of an analysis of bacterial resistance against the combination of antibiotics, in particular against amoxicillin and metronidazole. The diversity of the oral microflora, reaching up to 700 different bacterial species (Kazor *et al.*, 2003), makes it impossible to analyze every single bacterial strain regarding a genetic profile encoding for antibiotic resistance. In addition, the molecular mechanisms of bacterial resistance to antibiotics are quite far from being completely understood. Therefore, the antibiotic susceptibility of a subgingival plaque sample or of putative periodontal pathogens needs to be analyzed by conventional culture techniques (Armitage, 2003).

A major concern of the presented approach is the natural biofilm association of the subgingival bacterial samples analysed. A biofilm is a difficult therapeutic target because of its three-dimensional structure, which protects the bacteria from the host response as well as from antimicrobial agents (Socransky and Haffajee, 2002; Eick and Pfister, 2004). The methodology of the present report allowed the interactions between culturable microorganisms, but no attempt was made to mimic other characteristics of the subgingival plaque. Different results may be expected when a biofilm of mixed microbial samples is formed on an appropriate substrate prior to their susceptibility testing. However, such an approach is currently not available. The chosen methodology aims to provide an approach for clinically relevant susceptibility testing.

According to the contemporary understanding of the pathogenesis, periodontal diseases are caused by an opportunistic infection with a conglomerate of potentially periopathogenic microorganisms organized in the subgingival biofilm. A number of different test methods and procedures are available for qualitative and quantitative microbiological diagnostics of putative periopathogens. However, the pathogenic potential of a certain putative periodontal pathogen against the host can hitherto not be determined. Moreover, major individual differences in the immune response are caused by a number of acquired or genetic factors. Although specific bacteria have a periopathogenic potential or

may initiate periodontal inflammation, it is still difficult to determine the microbiota responsible for the onset and progression of disease in the individual subject. Thus, in the diagnosis and therapy of periodontal diseases, microbiological identification and susceptibility testing of single disease-associated strains may be of limited value (Mombelli *et al.*, 2002; Sanz *et al.*, 2004). Instead or in addition to the conventional approach of microbiological diagnostics, susceptibility testing of the entire subgingival plaque sample may offer additional valuable information for the choice of the antibiotic to be administered adjunctively.

Improved therapy outcomes indicate that patients with periodontal diseases - particularly those with highly destructive forms (aggressive and/or advanced) - may profit from an adjunctive antibiotic therapy using amoxicillin and metronidazole (Guerrero *et al.*, 2005; Kaner *et al.*, 2007a). However, due to the increased use of antibiotics and the alarming development of resistant strains, antibiotics should be administered with care, and testing the susceptibility of a given individual's microflora may have an increasing importance (Walker, 1996; Van Winkelhoff *et al.* 2005; Lakhssassi *et al.*, 2005; Walter and Weiger, 2006). Therefore, microbial testing can not be recommended for routine dental practise. However, some patients, in particular those in need of adjunctive antimicrobial therapy, may profit from the information about potential therapeutic targets (Armitage, 2003). Susceptibility testing of whole subgingival samples to metronidazole and amoxicillin and to their combination seems to offer a rational diagnostic tool to the selection of adjunctive antibiotic therapy. In the event of an unfavorable response, i.e. bacterial growth on agar plates supplemented with amoxicillin and metronidazole, another antibiotic has to be tested and subsequently applied for adjunctive antimicrobial therapy.

The current report about susceptibility analyses of subgingival plaque samples was initiated as a proof-of-principle study. The microbial results derived from the audit of four cases may indicate a potential benefit for further analysis in a larger clinical microbiological trial.

Acknowledgements

References

Armitage, G. C. Development of a classification system for periodontal diseases and conditions. *Annuals of Periodontology* 1999; **4**:1-6.

Armitage, G. C. Diagnosis of periodontal diseases. *Journal of Periodontology* 2003; **74**:1237-1247.

Buchmann, R., Nunn, M. E., Van Dyke, T. E. and Lange, D. E. Aggressive periodontitis: 5-year follow-up of treatment. *Journal of Periodontology* 2002; **73**:675-683.

Casas, A., Herrera, D., Martin-Carnes, J., Gonzalez, I., O'Connor, A. and Sanz, M. Influence of sampling strategy on microbiologic results before and after periodontal treatment. *Journal of Periodontology* 2007; **78**:1103-1112.

Clinical and Laboratory Standards Institute Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard M11-A7. CLSI, Payne, WA, USA. 2007.

Eick, S. and Pfister, W. Efficacy of antibiotics against periodontopathogenic bacteria within epithelial cells: an *in vitro* study. *Journal of Periodontology* 2004; **75**:1327-1334.

Gordon, J., Walker, C., Lamster, I. *et al.* Efficacy of clindamycin hydrochloride in refractory periodontitis. 12-month results. *Journal of Periodontology* 1985; **56**:75-80.

Guerrero, A., Griffiths, G. S., Nibali, L. *et al.* Adjunctive benefits of systemic amoxicillin and metronidazole in non-surgical treatment of generalized aggressive periodontitis: a randomized placebo-controlled clinical trial. *Journal of Clinical Periodontology* 2005; **32**:1096-1107.

Hanioka, T., Tanaka, M., Takaya, K., Matsumori, Y. and Shizukuishi, S. Pocket oxygen tension in smokers and non-smokers with periodontal disease. *Journal of Periodontology* 2000; **71**:550-554.

Kaner, D., Christan, C., Dietrich, T., Bernimoulin, J. P., Kleber, B. M. and Friedmann, A. Timing affects the clinical outcome of adjunctive systemic antibiotic therapy for generalized aggressive periodontitis. *Journal of Periodontology* 2007a; **78**:1201-1208.

Kaner, D., Bernimoulin, J. P., Hopfenmüller, W., Kleber, B. M. and Friedmann, A. Controlled-delivery chlorhexidine chip versus amoxicillin/metronidazole as adjunctive antimicrobial therapy for generalized aggressive periodontitis: a randomized controlled clinical trial. *Journal of Clinical Periodontology* 2007b; **34**:880-891.

Karbach, J., Callaway, A., Willershausen, B., Wagner, W., Geibel, M. A. and Al-Nawas, B. Antibiotic resistance testing of the total implant-associated micro-flora and its pure isolates. *European Journal of Medical Research* 2007; **12**:120-128.

Kazor, C. E., Mitchell, P. M., Lee, A. M., Stokes, L. N., Loesche, W. J., Dewhirst, F. E. and Paster, B. J. Diversity of bacterial populations on the tongue dorsa of patients with halitosis and healthy patients. *Journal of Clinical Microbiology* 2003; **41**:558-563.

Lakhssassi, N., Elhajoui, N., Lodter, J. P., Pineill, J. L. and Sixou, M. Antimicrobial susceptibility variation of 50 anaerobic periopathogens in aggressive periodontitis: an interindividual variability study. *Oral Microbiology and Immunology* 2005; **20**:244-252.

Lekovic, V., Kenney, E. B., Carranza, F.A. Jr. and Endres, B. The effect of metronidazole on human periodontal disease. A clinical and bacteriological study. *Journal of Periodontology* 1983; **54**:476-480.

Lindhe, J., Heijl, L., Goodson, J. M. and Socransky, S. S. Local tetracycline delivery using hollow fiber devices in periodontal therapy. *Journal of Clinical Periodontology* 1979; **6**:141-149.

Listgarten, M. A., Lindhe, J. and Hellden, L. Effect of tetracycline and/or scaling on human periodontal disease. Clinical, microbiological, and histological observations. *Journal of Clinical Periodontology* 1978; **5**:246-271.

Magnusson, I., Clark, W. B., Low, S. B., Maruniak, J., Marks, R. G. and Walker, C.B. Effect of non-surgical periodontal therapy combined with adjunctive antibiotics in subjects with "refractory" periodontal disease. (I). Clinical results. *Journal of Clinical Periodontology* 1989; **16**:647-653.

Matarazzo, F., Figueiredo, L. C., Cruz, S. E. B. , Faveri, M. and Feres, M. Clinical and microbiological benefits of systemic metronidazole and amoxicillin in the treatment of smokers with chronic periodontitis: a randomized placebo-controlled study. *Journal of Clinical Periodontology* 2008; **35**:885–896.

Meyer, J., Lallam-Laroye, C. and Dridi, M. Aggressive periodontitis - what exactly is it? *Journal Clinical Periodontology* 2004; **31**:586-587.

Mombelli, A., Casagni, F. and Madianos, P. N. Can presence or absence of periodontal pathogens distinguish between subjects with chronic and aggressive periodontitis? A systematic review. *Journal of Clinical Periodontology* 2002; **29** Suppl 3:10-21.

Needleman, I. G. and Watts, T.L. The effect of 1% metronidazole gel in routine maintenance of persistent furcation involvement in human beings. *Journal of Periodontology* 1989; **60**:699-703.

Pavicic, M. J., van Winkelhoff, A. J., Pavicic-Temming, Y. A. and de Graaff, J. Amoxycillin causes an enhanced uptake of metronidazole in *Actinobacillus actinomycetemcomitans*: a mechanism of synergy. *Journal of Antimicrobial Chemotherapy* 1994a; **34**:1047-1050.

Pavicic, M. J., van Winkelhoff, A. J., Douque, N. H., Steures, R. W. and de Graaff, J. Microbiological and clinical effects of metronidazole and amoxicillin in *Actinobacillus actinomycetemcomitans*-associated periodontitis. A 2-year evaluation. *Journal of Clinical Periodontology* 1994b; **21**:107-112.

Rooney, J., Wade, W. G., Sprague, S. V., Newcombe, R. G. and Addy, M. Adjunctive effects to non-surgical periodontal therapy of systemic metronidazole and amoxycillin alone and combined. A placebo-controlled study. *Journal of Clinical Periodontology* 2002; **29**:342-350.

Sanz, M., Lau, L., Herrera, D., Morillo, J. M. and Silva, A. Methods of detection of *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis and Tannerella forsythensis* in periodontal microbiology, with special emphasis on advanced molecular techniques: a review. *Journal of Clinical Periodontology* 2004; **31**:1034-1047.

Schacher, B., Baron, F., Rossberg, M., Wohlfeil, M., Arndt, R. and Eickholz, P. *Aggregatibacter actinomycetemcomitans* as indicator for aggressive periodontitis by two analysing strategies. *Journal of Clinical Periodontology* 2007; **34**:566-573.

Seifert, H. and Dalhoff, A. German multicentre survey of the antibiotic susceptibility of *Bacteroides fragilis* group and *Prevotella* species isolated from intra-abdominal infections: results from the PRISMA study. *Journal of Antimicrobial Chemotherapy* 2010; 65:2405-2410

Socransky, S. S. and Haffajee, A. D. Dental biofilms: difficult therapeutic targets. *Periodontology 2000* 2002; **28**:12-55.

Stabholz, A., Shapira, L., Mahler, D. *et al.* Using the PerioChip in treating adult periodontitis: an interim report. *Compendium of Continuing Education in Dentistry* 2000; **21**:325-8, 330-332.

van Winkelhoff, A. J., Rodenburg, J. P., Goene, R. J., Abbas, F., Winkel, E. G. and de Graaff, J. Metronidazole plus amoxycillin in the treatment of *Actinobacillus actinomycetemcomitans*-associated periodontitis. *Journal of Clinical Periodontology* 1989; **16**:128-131.

van Winkelhoff, A. J., Gonzales, D. H., Winkel, E. G., Dellemijn-Kippuw, N., Vandenbroucke-Grauls, C. M. and Sanz, M. Antimicrobial resistance in the subgingival microflora in patients with adult periodontitis. A comparison between The Netherlands and Spain. *Journal of Clinical Periodontology* 2000; **27**:79-86.

van Winkelhoff, A. J., Bosch-Tijhof, C. J., Winkel, E. G. and van der Reijden, W. A. Smoking affects the subgingival microflora in periodontitis. *Journal of Periodontology* 2001; **72**:666-671.

van Winkelhoff, A. J., Herrera, D., Oteo, A. and Sanz, M. Antimicrobial profiles of periodontal pathogens isolated from periodontitis patients in The Netherlands and Spain. *Journal of Clinical Periodontology* 2005; **32**:893-898.

van der Velden, U., Varoufaki, A., Hutter, J. W., *et al.* Effect of smoking and periodontal treatment on the subgingival microflora. *Journal of Clinical Periodontology* 2003; **30**:603-610.

Walker, C. B. The acquisition of antibiotic resistance in the periodontal microflora. *Periodontology 2000* 1996; **10**:79-88.

Walter, C. and Weiger, R. Antibiotics as the only therapy of untreated chronic periodontitis: a critical commentary. *Journal of Clinical Periodontology* 2006; **33**:938-939.

Warnakulasuriya, S., Dietrich, T., Bornstein, M. M., *et al.* Oral health risks of tobacco use and effects of cessation. *International Dental Journal* 2010; 60:7-30

Ximenez-Fyvie, L. A., Maguer-Flores, A., Jacobo-Soto, V., Lara-Cordoba, M., Moreno-Borjas, J. Y. and Cantara-Maruri, E. Subgingival microbiota of periodontally untreated Mexican subjects with generalized aggressive periodontitis. *Journal of Clinical Periodontology* 2006; **33**:869-877.