

# Antimicrobial profiles of periodontal pathogens isolated from periodontitis patients in the Netherlands and Spain

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## Abstract

**Background and Aim:** Antimicrobial resistance of periodontal pathogens towards currently used antibiotics in periodontics has been investigated in a previous study. Microbial resistance in the periodontal microflora was more frequently observed in Spanish patients in comparison with Dutch patients. The aim of the present study was to compare antimicrobial susceptibility profiles of five periodontal bacteria isolated from periodontitis patients in Spain and in the Netherlands.

**Material and Methods:** Subgingival plaque samples from adult patients with periodontitis were collected and cultured on selective and non-selective plates. *Actinobacillus actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Micromonas micros* were isolated and used for minimal inhibitory concentration tests using the Epsilon meter (E-test) technique. Eight different antibiotics were tested on all bacterial isolates. MIC<sub>50</sub> and MIC<sub>90</sub> values for each antibiotic and each species were determined and the percentage of resistant strains was calculated.

**Results:** Significantly higher MIC values were noted in Spanish strains of *F. nucleatum* for penicillin, ciprofloxacin, of *P. intermedia* for penicillin, amoxicillin and tetracycline, of *M. micros* for tetracycline, amoxicillin and azithromycin, and of *P. gingivalis* for tetracycline and ciprofloxacin. Based on breakpoint concentrations, a higher number of resistant strains in Spain were found in *F. nucleatum* for penicillin, amoxicillin and metronidazole, in *Prevotella intermedia* for tetracycline and amoxicillin, and in *A. actinomycetemcomitans* for amoxicillin and azithromycin. Resistance of *P. gingivalis* strains was not observed for any of the antibiotics tested both in Spain and the Netherlands.

**Conclusions:** Differences exist in the susceptibility profiles of periodontal pathogens isolated from periodontitis patients in Spain and in the Netherlands. This implicates that antibiotic susceptibility testing is necessary to determine efficacy of antimicrobial agents. Also, clinical studies with antibiotics should take these differences into account. The information from the present study indicates that it may not be possible to develop uniform protocols for usage of antibiotics in the treatment of severe periodontitis in the European Union.

Key words: antibiotic susceptibility; Epsilon meter test; periodontal pathogens; Spain; the Netherlands

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The amount of antibiotics used in the European Union shows great variation between countries. Cars et al. (2001) analysed data on non-hospital sales of

antibiotics for 1997 from 15 European countries. They found that the number of defined daily doses (DDD) per 1000 inhabitants per day varied more than

fourfold. France (DDD = 36.5), Spain (DDD = 32.4), Portugal (DDD = 28.8) and Belgium (DDD = 26.7) were countries with the highest sales, whereas,

Denmark (DDD = 11.3), Sweden (DDD = 13.5), Germany (DDD = 13.6) and the Netherlands (DDD = 8.9) showed the lowest sales. The higher usage of antimicrobial drugs in Mediterranean countries when compared with central and northern countries has been reported earlier (Baquero 1996). Also, the non-compliance rates have shown to be higher in Spain (42%), Italy (34%) and France (16%) in comparison with the UK (9%) (Pradier et al. 1997). As a consequence of this abusive usage and poor compliance, antimicrobial resistance in southern European countries is higher when compared with northern countries (Machka et al. 1988, Voss et al. 1994, Bronzwaer et al. 2004, Zinn et al. 2004). For instance, the prevalence of penicillin-resistant pneumococci was reported to be higher in Spain and in France in comparison with UK, Germany and Italy (Baquero 1996). The proportion of methicillin-resistant *Staphylococcus aureus* (MRSA) in various European countries shows great variation and ranges from 0.1% in Denmark and 1.5% in the Netherlands, to 30% in Spain, 34% in France, 34% in Italy and up to 53% in Greece (Voss et al. 1994, Veldhuijzen et al. 2000, Jones et al. 2003). Higher proportions of MRSA strains towards antibiotics other than methicillin were also observed in Spain in comparison with the Netherlands and amounted, respectively, to 84.7% versus 55.5% for ciprofloxacin, 96.8% versus 44.4% for clindamycin, and 96.8% versus 55.5% for erythromycin (Voss et al. 1994).

$\beta$ -lactamase production is a mechanism responsible for bacterial resistance towards  $\beta$ -lactam antibiotics. The prevalence of  $\beta$ -lactamase-producing *Haemophilus influenzae* serotype b in Spain was reported to be 63.6%, which was significantly higher than in the Netherlands (12%) (Machka et al. 1988). Multi-drug resistance and resistance towards all  $\beta$ -lactam antibiotics was reported for clinical pneumococci isolates in Spain more than 15 years ago (Appelbaum et al. 1992).

Data on antimicrobial resistance of different periodontal bacteria in different European countries are scarce. Using whole plaque samples from periodontitis patients from the Netherlands and from Spain, our groups investigated the bacterial susceptibility towards different antibiotics (van Winkelhoff et al. 2000). Blood agar plates containing breakpoint concentrations of penicillin,

amoxicillin, amoxicillin and clavunate, metronidazole, erythromycin, azithromycin, clindamycin and tetracycline were used to determine the proportion of subgingival plaque bacteria that were resistant to these antibiotics. Statistically significant higher bacterial resistance of these periodontal bacteria was reported for penicillin, amoxicillin, metronidazole, clindamycin and tetracycline in samples from Spanish patients when compared with samples from Dutch patients. The mean number of different bacterial species growing on the selective plates and the percentage of resistant strains were also higher in the Spanish group. When the frequency of occurrence of tetracycline-resistant periodontal pathogens was studied, five patients from the Spanish group harboured more than three tetracycline-resistant periodontal pathogens, whereas this was not observed in any of the Dutch patients. Also, the subgingival microflora from periodontitis patients harboured more  $\beta$ -lactamase-producing bacteria in Spain when compared with Dutch patients (Herrera et al. 2000).

The purpose of this study was to further investigate differential patterns of antimicrobial resistance between Spain and the Netherlands by comparing the antimicrobial susceptibility profiles of five periodontal bacteria isolated from periodontitis patients using the Epsilonometer (E-test) technique.

## Materials and Methods

### Patients

Fifty-four patients from the periodontal graduate clinic at the University Complutense, Madrid, and 26 from the Periodontal Clinic at ACTA, Amsterdam, were selected for this study. This patient population fulfilled the following selection criteria: (1) age >25 years; (2) destructive periodontal disease with pocket probing depth of  $\geq 5$  mm at  $\geq 3$  teeth in each quadrant of the dentition; (3) radiographic evidence of alveolar bone loss in each quadrant of the dentition; (4) no use of systemic or topical antimicrobial therapy. Once selected, each patient provided a pooled subgingival plaque sample.

### Microbiological sampling

In each quadrant of the dentition, the deepest pocket, showing bleeding on probing and with the maximum of

attachment loss was selected for microbiological sampling. After careful removal of supragingival plaque deposits, isolation of the sampling sites with cotton rolls and after gentle air-drying, two consecutive sterile paper points were inserted to the depth of the pockets and left in place for at least 10 s. Paper points from all four selected periodontal sites were pooled in 2.0 ml of reduced transport fluid (RTF) (Syed & Loesche 1972). Samples were processed within 2 h after sampling.

### Microbiological procedures

After vortexing for 30 s, samples were 10-fold serially diluted in RTF and 100  $\mu$ l of appropriate dilutions were plated on non-selective 5% horse blood agar plates (Oxoid no. 2, Oxoid Ltd., Basingstoke, UK) supplemented with haemin (5 mg/l) and menadione (1 mg/l). Samples were also plated onto trypticase soy serum-bacitracin-vancomycin plates (TSBV, Slots 1982) or on Dentaid-1 plates (Alsina et al. 2001) for selective isolation of *Actinobacillus actinomycetemcomitans*. Blood agar plates were incubated for up to 14 days at 37°C in 80% N<sub>2</sub>, 10% CO<sub>2</sub> and 10% H<sub>2</sub>. TSBV and Dentaid-1 plates were incubated in air plus 5% CO<sub>2</sub> at 37°C for 5 days.

The periodontal pathogens *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum* and *Micromonas micros* were identified using standard anaerobic techniques (van Winkelhoff et al. 1985, Winkel et al. 1997). *A. actinomycetemcomitans* was identified on the basis of its characteristic colony morphology (star-like inner structure), on the basis of a positive catalase reaction with 3% hydrogen peroxide and on a set of specific enzymes (APIZYM, BioMerieux, Boxtel, the Netherlands). Pure isolates were kept on plates, or were preserved at -70°C, until used for MIC determinations.

### Susceptibility testing

The minimal inhibitory concentrations for penicillin, amoxicillin, amoxicillin plus clavunate, tetracycline, clindamycin, ciprofloxacin, metronidazole and azithromycin were determined using the E-test (AB Biodisk, Solna, Sweden) (Citron et al. 1991, Nachnani et al. 1992). Bacterial strains were grown on blood agar plates (Oxoid no. 2, Basingstoke, UK) supplemented with 5% sheep

blood, haemin (5 mg/l) and menadione (1 mg/l) for 5 days. Then, test organisms were suspended in sterile phosphate-buffered saline equivalent to a 0.5 McFarland standard and streaked confluent over the surface of 150 mm diameter supplemented blood agar plates. In order to avoid drug interactions, only two strips were placed per plate. Plates were incubated in 80% N<sub>2</sub>, 10% CO<sub>2</sub> and 10% H<sub>2</sub> for 3 days. Inhibition zones were measured according to the recommendations of the manufacturer. All strains were tested in duplicate in two separate experiments. *Bacteroides fragilis* (ATCC 25285) was used as a reference strain for susceptibility testing.

#### Data analyses

The concentrations to which 50% and 90% of the strains were susceptible were defined as MIC<sub>50</sub> and MIC<sub>90</sub>, respectively. Results from each strain and each antibiotic were expressed in µg/ml, and were grouped by country. Quantitative results were compared using the Wilcoxon test.

Qualitative results were obtained by comparing each strain with a pre-defined breakpoint. When the value was lower, a strain was considered susceptible. Conversely, it was considered resistant if it was equal to or higher than the breakpoint. The pre-defined breakpoints were as follows: penicillin G, 0.5 µg/ml; amoxicillin, 3 µg/ml; amoxicillin plus clavulanate, 3.0+0.5 µg/ml; metronidazole, 8 µg/ml; tetracycline, 8 µg/ml; ciprofloxacin, 4 µg/ml; clindamycin, 4 µg/ml; and azithromycin, 2 µg/ml. The percentage of resistant strains was calculated for each pathogen and antimicrobial drug, and then comparisons were made between countries using the  $\chi^2$  test.

#### Results

The MIC values of the reference *B. fragilis* strain were all in the expected range.

#### Comparison of quantitative results

Table 1 summarizes the MIC<sub>50</sub> and MIC<sub>90</sub> values of five periodontal pathogens isolated from Spain and the Netherlands against a selected group of antibiotics. Penicillin G demonstrated significantly higher MIC values in Spanish isolates for *F. nucleatum* ( $p = 0.002$ )

and *P. intermedia* ( $p = 0.011$ ). The MIC values of amoxicillin were higher in Spanish isolates for *P. intermedia* ( $p = 0.002$ ) and *M. micros* ( $p = 0.012$ ) strains. The MIC values for clindamycin of *M. micros* were higher for Dutch isolates ( $p < 0.001$ ). The MIC values for tetracycline were higher in Spanish isolates for *P. intermedia* ( $p = 0.003$ ), *M. micros* ( $p = 0.029$ ) and *P. gingivalis* ( $p < 0.001$ ) strains. Significantly higher MIC values were observed for ciprofloxacin in Dutch isolates for *F. nucleatum* ( $p = 0.035$ ), and in Spanish isolates for *P. gingivalis* ( $p = 0.002$ ). Azithromycin demonstrated significantly higher MIC values in Spanish isolates for *M. micros* ( $p = 0.005$ ). No significant differences were detected for metronidazole and amoxicillin plus clavulanate.

#### Percentage of resistant strains

Table 2 shows the total number of strains, the number of susceptible strains and the percentage of resistant strains. A significantly higher number of *F. nucleatum* strains from Spain showed resistance for penicillin G ( $p = 0.038$ ), amoxicillin ( $p = 0.038$ ) and metronidazole ( $p = 0.043$ ). The percentage of *P. intermedia* strains resistant to tetracycline ( $p = 0.038$ ) and amoxicillin ( $p = 0.012$ ) was higher in Spain. Some *M. micros* strains demonstrated resistance to azithromycin (three Dutch and two Spanish strains), and one Spanish strain was resistant to metronidazole. *A. actinomycetemcomitans* showed a high level of resistance to penicillin G, clindamycin and metronidazole in at least 20% of Dutch and Spanish strains. In addition, one Spanish strain was resistant to ciprofloxacin and amoxicillin+clavulanate, and three strains were resistant to azithromycin. Statistically significant differences between countries were detected for amoxicillin ( $p = 0.013$ ) and azithromycin ( $p = 0.009$ ). *P. gingivalis* strains from both countries were susceptible to all antibiotics tested.

#### Discussion

In a previous study, we demonstrated that bacterial resistance in subgingival plaque samples taken from adult periodontitis patients against a number of common antibiotics was higher in Spain than in the Netherlands (van Winkelhoff et al. 2000). In that study, whole subgingival plaque samples were plated on

antibiotic-containing blood agar plates and the level of resistance was expressed as the percentage of the microflora growing on plates containing antibiotics in concentrations above the breakpoint concentration of a given antimicrobial agent. A higher level of resistance in Spain was found for penicillin, amoxicillin, metronidazole, clindamycin and tetracycline. In the present study, selected periodontal bacterial species were isolated from subgingival plaque samples from adult patients with destructive periodontal disease and subjected to minimal inhibitory concentration tests. For this objective, strains of *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *M. micros* and *F. nucleatum* were selected.

We used the E-test, as this technique has shown to be reliable in the determination of MIC values of anaerobic bacteria (Citron et al. 1991, Nachnani et al. 1992). The results from the present study confirm our previous observations that several Spanish bacterial species isolated from periodontal lesions demonstrated higher MIC values when compared with Dutch isolates. Differences were observed for the  $\beta$ -lactam antibiotics, such as penicillin and amoxicillin, against *P. intermedia*, *F. nucleatum* and *A. actinomycetemcomitans* strains. Production of  $\beta$ -lactamase has been reported in the subgingival microflora from periodontitis patients (van Winkelhoff et al. 1997) and this production was found more frequently in Spanish patients when compared with Dutch patients (Herrera et al. 2000). This was corroborated in this study by showing that protected amoxicillin (with clavulanic acid) showed a significant improvement of the MIC<sub>90</sub> values in comparison with unprotected amoxicillin. For tetracycline, the MIC<sub>90</sub> values were higher for all species isolated from Spanish samples. Metronidazole is active against a wide range of anaerobic bacterial species. As the subgingival microflora in periodontitis is dominated by strict anaerobic species, it is a common antibiotic in the treatment of severe periodontitis and it is often the first drug of choice. The majority of test species showed good susceptibility towards this drug, with the exception of *A. actinomycetemcomitans*, which is not a strict anaerobic species. However, it is known that the hydroxy metabolite of metronidazole is three to four times more active against *A. actinomycetemcomitans* (Pavićić et al. 1992) and that it

Table 1. The mean MIC50 ( $\mu\text{g/ml}$ ) and MIC90 ( $\mu\text{g/ml}$ ) of five periodontal pathogens isolated from periodontitis patients from SP and NL towards selected antibiotics

	Penicillin		Amoxicillin		Amoxicillin+clavunilate		Tetracycline	
	SP	NL	SP	NL	SP	NL	SP	NL
<i>A. actinomycetemcomitans</i>								
Range	0.094–256	0.047–1.5	0.064–32	0.032–0.75	0.094–6	0.02–0.75	0.001–2	0.015–0.55
MIC50	0.5	0.5	0.25	0.25	0.25	0.25	0.064	0.125
MIC90	1	1	32	0.38	1.5	0.5	0.38	0.19
<i>P. gingivalis</i>								
Range	<0.016	<0.016	<0.016	<0.016	<0.016	<0.016	0.25–1*	0.015–0.32
MIN50	<0.016	<0.016	<0.016	<0.016	<0.016	<0.016	0.75	0.015
MIC90	<0.016	<0.016	<0.016	<0.016	<0.016	<0.016	0.5	0.023
<i>P. intermedia</i>								
Range	0.015–56*	0.001–33	0.015–56*	0.015–5	0.015–0.5	0.015–0.05	0.015–8*	0.015–3
MIC50	0.015	0.015	0.25	0.015	0.015	0.015	1.5	0.032
MIC90	24	5	256	1.5	0.25	0.032	8	2
<i>F. nucleatum</i>								
Range	0.015–56*	0.001–0.064	0.015–256	0.05–0.094	0.015–0.25	0.015–0.064	0.015–0.47	0.015–1
MIC50	0.015	0.008	0.015	0.015	0.015	0.015	0.032	0.04
MIC90	1	0.015	256	0.023	0.125	0.023	0.064	0.5
<i>M. micros</i>								
Range	<0.016	<0.016	0.015–0.75*	0.015–0.125	<0.016	0.015–0.125	0.015–0.75*	0.015–0.047
MIC50	<0.016	<0.016	0.015	0.015	<0.016	0.015	0.015	0.023
MIC90	<0.016	<0.016	0.015	0.047	<0.016	0.032	0.25	0.032
	Clindamycin		Ciprofloxacin		Metronidazole		Azithromycin	
	SP	NL	SP	NL	SP	NL	SP	NL
<i>A. actinomycetemcomitans</i>								
Range	0.016–256	0.04–5	0.001–32	0.001–0.094	0.05–256	0.125–256	0.19–256	0.047–1
MIC50	1.5	1.5	0.001	0.003	48	1	1	0.38
MIC90	8	4	2	0.008	256	64	3	0.875
<i>P. gingivalis</i>								
Range	<0.016	<0.016	0.15–0.75*	0.001–2	<0.016	<0.016	<0.016	0.015–1.5
MIN50	<0.016	<0.016	0.25	0.125	<0.016	<0.016	<0.016	0.25
MIC90	<0.016	<0.016	0.75	0.38	<0.016	<0.016	<0.016	0.5
<i>P. intermedia</i>								
Range	0.015–256	<0.016	0.001–0.64	0.047–0.58	0.015–0.19	0.015–0.047	0.015–256	0.015–0.94
MIC50	0.015	<0.016	0.19	0.19	0.015	0.015	0.094	0.064
MIC90	0.015	<0.016	0.38	0.38	0.125	0.032	1.5	0.38
<i>F. nucleatum</i>								
Range	0.015–256	0.015–0.575	0.001–1	0.25–4*	0.015–256	0.015–0.023	0.094–256	0.047–8
MIC50	0.015	0.015	0.5	1	0.015	0.015	0.25	0.23
MIC90	0.75	0.047	1	1.5	256	0.016	1.5	2
<i>M. micros</i>								
Range	0.015–0.64	0.015–0.5*	0.001–0.94	0.094–0.5	0.015–256	0.015–0.25	0.19–256*	0.015–256
MIC50	0.015	0.22	0.125	0.16	0.015	0.015	0.5	1
MIC90	0.094	0.38	0.38	0.38	0.015	0.125	1	2

\*Statistically significant higher value ( $p < 0.05$ , Wilcoxon's test).

SP, Spain; NL, the Netherlands.

acts synergistically with amoxicillin against this pathogen (Pavičić et al. 1991). For this reason, metronidazole in combination with amoxicillin has been shown to be effective in the treatment of *A. actinomycetemcomitans*-associated periodontal disease (van Winkelhoff et al. 1989, Berglundh et al. 1998, Winkel et al. 2001). However, the MIC90 value of  $> 256 \mu\text{g/ml}$  of the Spanish *A. actinomycetemcomitans* may indicate that the clinical efficacy of this combination therapy may not be identi-

cal in Spain as it is in other European countries.

The higher MIC values among Spanish bacterial isolates and the higher percentage of resistant periodontal bacterial strains is most likely because of the higher antibiotic consumption (Baquero 1996, Cars et al. 2001) and poor compliance to the medication (Pradier et al. 1997) in Spain. This study provides evidence that supports the view that standard guidelines for antimicrobial use in the treatment of periodontal infec-

tions should not be recommended, as major differences in the antimicrobial profile of major periodontal pathogens were found when studied in two European countries. Antimicrobial susceptibility testing may therefore be needed as an integrated step in the microbial diagnosis of severe periodontitis patients. Moreover, further studies are needed in other countries of the European Union to investigate these geographical variations in the antimicrobial drug resistance of the periodontal microflora.

Table 2. Number of strains of five periodontal pathogens from NL and SP susceptible and resistant towards PG, CM, TC, CL, XL, AC and AZ based on selected breakpoint values

	PG		CM		TC		CL		MZ		XL		AC		AZ	
	NL	SP	NL	SP	NL	SP	NL	SP	NL	SP	NL	SP	NL	SP	NL	SP
<i>F. nucleatum</i>																
n susceptible	20	8	20	9	20	10	19	10	19	8	20	10	20	8	18	9
n total	20	10	20	10	20	10	20	10	19	10	20	10	20	10	20	10
% resistant	0.0	20.0*	0.0	10.0	0.0	0.0	5.0	0.0	0.0	20.0*	0.0	0.0	0.0	20.0*	10.0	10.0
<i>P. intermedia</i>																
n susceptible	18	11	24	16	24	15	24	18	24	18	24	18	23	12	24	16
n total	24	18	24	18	24	18	24	18	24	18	24	18	24	18	24	18
% resistant	25.0	38.9	0.0	11.1	0.0	16.7*	0.0	0.0	0.0	0.0	0.0	0.0	4.2	33.3*	0.0	11.1
<i>M. micros</i>																
n susceptible	23	19	23	19	22	19	23	19	23	18	23	19	23	19	20	17
n total	23	19	23	19	22	19	23	19	23	19	23	19	23	19	23	19
% resistant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.3	0.0	0.0	0.0	0.0	13.0	10.5
<i>A. actinomycetemcomitans</i>																
n susceptible	8	4	14	7	18	10	18	9	13	4	18	9	18	6	18	6
n total	18	10	18	10	18	10	18	10	18	10	18	10	18	9	18	9
% resistant	55.6	60.0	22.2	30.0	0.0	0.0	0.0	10.0	27.8	60.0	0.0	10.0	0.0	33.3*	0.0	33.3*
<i>P. gingivalis</i>																
n susceptible	26	15	26	15	26	15	26	15	26	15	26	15	26	15	26	15
n total	26	15	26	15	26	15	26	15	26	15	26	15	26	15	26	15
% resistant	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

\*Statistically significant higher value ( $p < 0.05$ ,  $\chi^2$ ).

Selected breakpoint values: PG, 0.5 µg/ml; AC, 3 µg/ml; XL, 3.0+0.5 µg/ml; MZ, 8 µg/ml; TC, 8 µg/ml; CL, 4 µg/ml; CM, 4 µg/ml; AZ, 2 µg/ml.

PG, penicillin; CM, clindamycin; TC, tetracycline; CL, ciprofloxacin; XL, amoxicillin+clavulanate; AC, amoxicillin; AZ, azithromycin; SP, Spain; NL, the Netherlands.

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**Clinical Relevance**

*Scientific rationale for the study:* Antibiotic resistance among human pathogens is higher in Southern European countries and is related to a high intake of these drugs. We studied antibiotic resistance among periodontal pathogens isolated in the Netherlands and Spain.

*Principal findings:* Periodontal pathogens from Spain showed higher minimal inhibitory concentrations for multiple antibiotics in comparison with Dutch isolates. The percentage of resistant periodontal strains was significantly higher for most species tested in Spain.

*Practical implications:* The findings of this study indicate that a uniform adjunctive systemic antimicrobial therapy to treat severe periodontitis may not be possible and antimicrobial susceptibility testing may be necessary for a predictable treatment outcome.

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