Reduced susceptibility to amoxicillin of oral streptococci following amoxicillin exposure

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As antibiotic pressure often triggers bacterial resistance, the use of short-duration therapies is increasingly recommended. The objective of the present study was to evaluate both the clinical efficiency and the impact on oral streptococci of a 3 day versus a 7 day amoxicillin therapy for odontogenic infection requiring tooth extraction. On day 0, patients were randomly assigned to a 3 day or 7 day amoxicillin treatment. The tooth was extracted on day 2 and the post-operative follow-up was carried out on day 9. Oral flora was collected on days 0, 9 and 30, and the susceptibility of the streptococci to amoxicillin was determined. The results showed that treatment with amoxicillin for 3 or 7 days had a similar clinical efficiency, and also induced similar selection of oral streptococci with reduced susceptibility to amoxicillin, suggesting that the selection of strains with reduced susceptibility to amoxicillin is a rapid phenomenon, appearing even with short-duration therapies.

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INTRODUCTION

Antibiotic resistance is most prevalent in countries with a high level of antibiotic consumption, supporting the link between antibiotic use and the emergence of bacterial resistance (Foucault & Brouqui, 2007; Livermore, 2005). The antibiotic resistance of oral and gut flora may be due to the selection of clones with reduced susceptibility during antibiotic treatment, but also to the transfer of resistance genes from exogenous bacteria (Beovic, 2006; Dogan *et al.*, 2005; Wang *et al.*, 2006). Horizontal transfer of antibiotic resistance genes may occur between food-borne bacteria and the oral microbiota (Wang *et al.*, 2006), but also between commensals and pathogens. With the observation of antibiotic resistance gene transfers between oral streptococci and *Streptococcus pyogenes* and *Streptococcus pneumoniae* (Cerdá Zolezzi *et al.*, 2004; Dowson *et al.*, 1990; Jönsson & Swedberg, 2006; Nakayama & Takao, 2003), there is increasing evidence that the commensal flora can serve as a reservoir of antibiotic resistance genes. Thus, it is important to focus not only on the resistance of

pathogens but also on that of commensals (Andremont, 2003; Bryskier, 2002).

Medical misuse of antibiotics is one of the factors for the development of resistance. Therefore, reducing the use of antibiotics and defining guidelines for optimal prescriptions are encouraged (Foucault & Brouqui, 2007; Hamilton-Miller, 2004; Levy, 2001). A growing consensus favours the combination of high doses and short-duration therapies that are less selective for resistance than long, low-dose regimens (Guillemot *et al.*, 1998; Livermore, 2007). Amoxicillin is currently used in dental practice. Amoxicillin prescriptions for odontogenic infections follow professional guidelines (AFSSAPS, 2002; ANDEM, 1996) and the recommended doses of amoxicillin are 1 g every 12 h or 500 mg every 8 h, for a period of time varying from 5 to 10 days (AFSSAPS, 2002; Bascones *et al.*, 2004; Poveda-Roda *et al.*, 2007).

Although several reports have described the emergence of antibiotic resistance among viridans streptococci of neutropenic patients (Bruckner & Gigliotti, 2006; Ombanda-Moussa *et al.*, 2002; Prabhu *et al.*, 2005), very little is known about this phenomenon in the general healthy population. The objective of our study was to evaluate the susceptibility to amoxicillin of oral

Abbreviations: AFSSAPS, Agence Française de Sécurité Sanitaire des Produits de Santé; AP-HP, Assistance Publique Hôpitaux de Paris; CI, confidence interval; PBP, penicillin-binding protein.

streptococci in patients with an odontogenic infection requiring tooth extraction and receiving amoxicillin therapy for 3 or 7 days.

METHODS

Patients. Patients were eligible for the study if they had an indication of tooth extraction associated with amoxicillin monotherapy according to the good practice rules on antibiotherapy in odontology and stomatology defined by the Agence Française de Sécurité Sanitaire des Produits de Santé (AFSSAPS, 2002). Patients were excluded if they had taken any antibiotic within the previous 45 days, if they had a special infectious risk or in any case of amoxicillin contraindication. Pregnant or breastfeeding women were also excluded. Consequently, 81 patients in good health, aged 19–45 years, were recruited from emergency dental consultations of three university hospitals.

Written informed consent was obtained from all participants. The protocol of this study was approved by the ethics committee of Henri Mondor Hospital (Créteil, France).

Study design. Group 1 received a 3 day amoxicillin therapy (1 g amoxicillin twice daily orally) followed by a 4 day placebo treatment period. Group 2 received a standard 7 day amoxicillin therapy. A computer-generated randomization list was drawn up by an independent statistician using blocking. Amoxicillin treatment started on the day of inclusion in the study (day 0). Dentists and patients were blind to the treatment assignment for the duration of the study. The infected tooth was extracted 2 days after the beginning of the antibiotic treatment (day 2), and the post-operative follow-up was carried out 1 week after tooth extraction (day 9). An additional follow-up was planned 1 month later (day 30).

Clinical parameters collected on day 9 were evaluated according to the following criteria: (i) pain was evaluated by its intensity during the days following surgery using an analogue visual scale varying from 0 (no pain) to 10 (very intense pain), and by the total amount (in mg) of paracetamol ingested, and (ii) the infectious state was evaluated with respect to local wound healing, regional adenopathy and fever. The wound healing score combined local inflammation and sensitivity, and the presence or absence of a blood clot.

Bacteriological analysis. Oral flora was collected on days 0, 9 and 30 with a sterile cotton swab from the buccal side of the gingiva between the two mandibular canines. The swabs were immediately dispersed in 1 ml sterile 0.9% NaCl solution, and the samples were transferred to the microbiology department of Hôpital de l'Hôtel-Dieu.

Neat and diluted (10^{-1} , 10^{-2} , 10^{-3} and 10^{-4}) samples were cultivated for 48 h at 37 °C under 5% CO₂ on 5% sheep blood Columbia agar (bioMérieux) containing colistin (10 mg l⁻¹), nalidixic acid (15 mg l⁻¹) and amphotericin B (2 mg l⁻¹), with the addition of 0, 0.5, 2 or 16 mg amoxicillin l⁻¹. Gram stain, catalase activity, pyrrolidonyl arylamidase disc and optochin susceptibility tests were carried out on each type of α -haemolytic or non-haemolytic colony. The colonies of oral streptococci (Gram-positive cocci arranged in pairs or as chains, catalase negative, pyrrolidonyl arylamidase negative and optochin resistant) were numbered and studied further. The isolates were initially identified by use of a Rapid ID 32 Strep strip (bioMérieux) as recommended by the manufacturer. Identification was confirmed by sequencing the 16S rRNA and manganese-dependent superoxide dismutase-encoding (*sod A*) genes as described previously (Brosius *et al.*, 1981; Poyart *et al.*, 1998). Amoxicillin MICs of the isolates were determined by an agar elsewhere method using an Etest (AB Biodisk) on Mueller–Hinton agar with 5% sheep blood according to the

guidelines of the Antibiogram Committee of the French Society for Microbiology (<http://www.sfm.asso.fr>). A streptococcal isolate with reduced susceptibility to amoxicillin was classified as 'intermediate' when the MIC ranged from >0.5 to 16 mg l⁻¹, and as 'resistant' when the MIC was >16 mg l⁻¹.

Statistical analysis. The analysis was by intention to treat. A 3 day amoxicillin therapy was considered to be non-inferior to 7 day therapy if the difference between treatments in the composite score was less than 2 points: the upper bound of the one-sided 95% confidence interval (CI) for the treatment difference had to be below the 2 points margin to declare that non-inferiority had been shown, with a significance level of 0.005. The non-inferiority margin was also 2 points for pain intensity during the post-operative week and was 2 g for the total amount of paracetamol ingested.

In each group, the susceptibility to amoxicillin was evaluated by the proportion of streptococci with reduced susceptibility out of the total number of streptococci. Associated 95% CIs were computed using the bootstrap percentile method to take into account the positive intra-patient correlation. Analyses were performed on the complete-case population.

Analyses were performed using SAS software. *P* values less than 0.005 were deemed significant, unless stated otherwise.

RESULTS AND DISCUSSION

On day 0, 81 patients were included in the study and randomized (Table 1). The mean age was 30 ± 11 years in group 1 and 33 ± 12 years in group 2. Of the 74 patients undergoing surgery on day 2, 56 were consulted on day 9 and evaluated for the clinical parameters. Fifty patients were analysed for amoxicillin susceptibility of their oral streptococci on day 0. A total of 33 patients of these 50 were followed up on day 9, and 26 of these 33 were also followed up on day 30 (Table 1).

Clinical parameters were not significantly different between the two groups of patients. Pain intensity and duration, as well as the total amount of ingested paracetamol, were similar in both groups. Regional adenopathy was noted for

Table 1. Flow of patients through each stage of the trial

The number of patients analysed for amoxicillin susceptibility of oral streptococci is indicated in parentheses.

Stage of trial	No. of patients		
	Total	Group 1: amoxicillin for 3 days + placebo for 4 days	Group 2: amoxicillin for 7 days
Inclusion (day 0)	81 (50)	42 (25)	39 (25)
Surgery (day 2)	74	40	34
Post-operative follow-up (day 9)	56 (33)	29 (17)	27 (16)
Further follow-up (day 30)	41 (26)	22 (15)	19 (11)

Table 2. Clinical non-inferiority [median (25th percentile, 75th percentile)] of 3 versus 7 day amoxicillin therapy

The experimental treatment was considered to be non-inferior to the regular treatment if the difference between treatments was less than a given margin, which was defined a priori. In other words, the upper bound of the one-sided 95 % CI for the treatment difference had to be below that margin to declare that non-inferiority had been shown, with a significance level of 0.005. The non-inferiority margin was 2 points for intensity of pain and wound healing scores, and 2 g for the total amount of paracetamol ingested.

Clinical parameter	Amoxicillin for 3 days + placebo for 4 days	Amoxicillin for 7 days	95 % CI
Intensity of pain	3.5 (3, 6)	4 (2, 6)	0 (-1, 2)
Total amount of paracetamol ingested (mg)	5000 (1600, 9000)	4000 (1000, 6000)	1 (-2, 3)
Wound healing score	1 (1, 2)	1 (1, 2)	0 (0, 1)

one patient in each group and the wound healing score was similar in both groups (Table 2).

In the group treated with amoxicillin for 3 days, the proportion of streptococci with reduced susceptibility to amoxicillin varied from 1.3 % of the total streptococci on day 0 to 23 % on day 9, and 7.7 % on day 30. The same evolution trend was observed for patients treated with amoxicillin for 7 days: streptococci with reduced susceptibility to amoxicillin comprised 1.7 % on day 0, 24.7 % on day 9 and 7 % on day 30 (Table 3).

In both groups, the development of streptococci with reduced susceptibility to amoxicillin varied greatly among patients (Fig. 1). Before amoxicillin treatment (day 0), these streptococci were isolated from almost all patients. However, they represented a very small proportion of the total streptococcal flora, usually less than 5 %. After amoxicillin treatment, the proportion increased, and for a few patients, these isolates represented the dominant flora on day 9. In most cases, the proportion of streptococci with

reduced susceptibility decreased between days 9 and 30, almost reaching the initial level.

Growing evidence shows that short-duration treatments reduce the impact of antibiotics on the commensal flora, minimizing the exposure of bacteria and thus the risk of emergence of resistance (Canet & Garau, 2002). Several studies that evaluated the link between treatment duration and the risk of carriage of penicillin-resistant pneumococci have argued in favour of a 5–7 day treatment (Guillemot *et al.*, 1998; Nasrin *et al.*, 2002; Schrag *et al.*, 2001). Similarly, recent studies have shown that a 7 day treatment with amoxicillin and clavulanic acid increased the number of oropharyngeal streptococci with decreased susceptibility to amoxicillin (Crémieux *et al.*, 2003). Our results indicated that 3 or 7 days of amoxicillin therapy demonstrated a similar clinical efficiency. In addition, the present data also suggest that both amoxicillin treatment durations induced a similar selection of oral streptococci with reduced susceptibility to this antibiotic.

The MICs of isolates with reduced susceptibility increased after amoxicillin therapy. On day 0, most of the isolates had MICs of $<3 \text{ mg l}^{-1}$. After amoxicillin therapy, the MICs increased and most of the clones displayed MICs between 3 and 16 mg l^{-1} . In addition, the number of resistant isolates with MICs of $>16 \text{ mg l}^{-1}$ increased (Fig. 2).

In streptococcal species, reduced susceptibility to penicillin results from altered penicillin-binding proteins (PBPs) that have a low affinity for penicillin. *S. pneumoniae*, the most studied streptococcal species, has six major PBPs: PBP1a, PBP1b and PBP2a (class A), PBP2b and PBP2x (class B) and PBP3 (class C) (Sauvage *et al.*, 2008). Susceptibility to penicillin can decrease progressively with the successive acquisition of modified PBPs by lateral gene transfer. The lateral transfer of altered *pbp* genes has been demonstrated to be intraspecific between various strains of *S. pneumoniae* (Stanhope *et al.*, 2007) and interspecific between *S. pneumoniae*, *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus sanguinis* (Chi *et al.*, 2007; Dowson *et al.*, 1990, 1993). The acquisition of resistance by *S. pneumoniae* is suggested to result from the following scenario:

Table 3. Overall percentage of streptococci isolates with reduced susceptibility to amoxicillin in the treatment groups

The total number of oral streptococci collected from the various patients from each group is indicated in the left column. The overall percentage was determined as the number of streptococci with reduced susceptibility/total number of streptococci. Associated 95 % CIs were computed using a bootstrap percentile method to take into account the positive intra-patient correlation.

Day	Group 1: amoxicillin 3 days + 4 days placebo		Group 2: amoxicillin 7 days	
	Total no. of streptococci	Percentage streptococci with reduced susceptibility (95 % CI)	Total no. of streptococci	Percentage streptococci with reduced susceptibility (95 % CI)
0	117.5×10^6	1.3 (0.5, 2.8)	238.8×10^6	1.7 (1.0, 3.8)
9	48.6×10^6	23.0 (14.6, 39.8)	54.8×10^6	24.7 (8.3, 70.6)
30	9.5×10^6	7.7 (3.4, 15.3)	39.2×10^6	7.0 (1.1, 8.3)

Fig. 1. Evolution of streptococci with reduced susceptibility to amoxicillin after (a) 3 day and (b) 7 day treatment. Each line represents the individual evolution of streptococci from one patient. The percentage of streptococci with reduced susceptibility to amoxicillin out of the total number of streptococci was evaluated on day 0 (before amoxicillin therapy), day 9 (after 3 or 7 days of amoxicillin treatment) and on day 30.

Fig. 2. Evolution of MICs in the two treatment groups: (a) 3 day and (b) 7 day treatment. For each patient harbouring streptococci with reduced susceptibility to amoxicillin, the MICs of five isolates were determined by Etest on days 0 (i), 9 (ii) and 30 (iii). The graphs represent the total count of isolates for each MIC.

commensal streptococci with point mutations of *pbp* genes, and thus reduced susceptibility, are selected by exposure to penicillin. Subsequently, the altered *pbp* genes are transferred to related streptococcal species, including *S. pneumoniae*. The susceptibility to penicillin decreases progressively and can lead to high degrees of resistance (Zapun *et al.*, 2008). The highest degree of resistance of *S. pneumoniae* to penicillins is due to concomitant alterations in PBP2x, PBP2b and PBP1a (Barcus *et al.*, 1995).

In Japan, *S. mitis* strains with reduced susceptibility to ampicillin (MICs of 1–8 mg l⁻¹) were isolated from the saliva of 52% healthy adults, with an accumulation of mutations in their *pbp* genes and alterations of the high-molecular-mass PBP1a, PBP2b and PBP2x (Nakayama & Takao, 2003). In the present study, we observed that the proportion of oral streptococci with reduced susceptibility increased under amoxicillin therapy. Resistant streptococci appeared similarly in the 3 and 7 day treatment groups. A total of 2 of the 33 patients followed up on day 9 for amoxicillin susceptibility harboured resistant streptococci before amoxicillin therapy. After antibiotic treatment, five additional patients had resistant streptococci. One month later, resistant isolates were still present in one patient, whereas they were not detectable in three patients, who harboured only intermediate streptococci. Resistant isolates with amoxicillin MICs of >16 mg l⁻¹ were identified as *S. mitis*, *S. oralis*, *Streptococcus parasanguinis*, *Streptococcus australis* and *Streptococcus infantis*. Published studies from Finland (Seppälä *et al.*, 2003) and Spain (Tomás *et al.*, 2004) also showed that most viridans streptococci with reduced susceptibility to penicillin or amoxicillin belong to *S. mitis* and *S. oralis* species.

In conclusion, both amoxicillin treatment durations induced the selection of oral streptococci with reduced susceptibility to this antibiotic. However, the similar clinical efficiency observed favours a reduction in the duration of antibiotic exposure. Moreover, as the transfer of resistance genes from oral streptococci to *S. pneumoniae* is known to occur, a survey of oral streptococcal flora would be an easy and useful indicator to evaluate the risk of penicillin resistance of pneumococci in the general population.

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