Emergence of antibiotic resistant *Streptococcus sanguis* in dental plaque of children after frequent antibiotic therapy

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Abstract

***Purpose**: In the pediatric population, several different antibiotic regimens are currently recommended for the treatment of otitis media. This study investigated whether therapy for otitis media was associated with the emergence of antibiotic-resistant oral bacteria.*

***Methods**: Streptococcus sanguis (S. sanguis) was isolated from supragingival dental plaque of children after a recent course of antibiotic. The isolated strains were tested for resistance to penicillin, amoxicillin, trimethoprim-sulfamethoxazole, and erythromycin and compared to isolated strains from age- and sex-matched control subjects, who had received no antibiotics within two years before sampling.*

***Results**: While control subjects harbored no resistant strains of S. sanguis, about 60% of children who had received antibiotics harbored S. sanguis which were resistant to at least one of the tested antibiotics. Nearly half of these strains were resistant to two or more antibiotics. Resistance to penicillin and amoxicillin decreased with the age of the child and with the length of time since exposure to the antibiotic. However, resistance to trimethoprim-sulfamethoxazole or erythromycin showed no relationship to the age of the child or the length of time since exposure to the antibiotic.*

***Conclusion**: The data show that children who had been treated for otitis media with common antibiotic protocols do harbor antibiotic-resistant oral streptococci which may complicate prophylactic and therapeutic regimens for bacterial endocarditis. (Pediatr Dent 21:181–185, 1999)*

The viridans group streptococci is a major taxon in the human oral flora. Residing principally in dental plaque, members of this commensal group have been associated with oral lesions such as periapical abscesses and periodontal infections.[1, 2] These alpha-hemolytic organisms are, however, considered to be of low virulence in the oral cavity. However, following dental treatment, normal oral hygiene procedures, or merely chewing food, subclinical streptococcal bacteremias occur.[3]

In the circulation, *Streptococcus sanguis (S. sanguis)* have been associated with systemic disorders involving platelets, particularly infective endocarditis.[4] Endocarditis infrequently affects children and young adults.[5–7] Nonetheless, children with congenital or acquired heart disease have a substantial lifetime risk for development of endocarditis. Among children with aortic stenosis and ventricular septal defect, the risk of endocarditis during the first decade of life has been estimated to be 3.2%.[8]

Although responsible for a declining proportion of cases of endocarditis, streptococci are still the most common cause, with the viridans group isolated in approximately 40% of patients.[9] The evidence that dental procedures cause endocarditis is circumstantial and it is difficult to prove that a particular procedure induced the bacteremia that caused an individual case of endocarditis.[10] However, it is presumed that between 15% to 50% of the cases of viridans streptococcal endocarditis are secondary to transient bacteremias originating from dental manipulation or oral infections.[11] Antibiotic prophylaxis for bacterial endocarditis is recommended by the American Heart Association and the American Dental Association in a standard protocol indicated for all dental procedures likely to cause bacteremia.[12] Despite periodically revised antibiotic prophylaxis protocols,[12] and advances in antibiotic therapy, bacterial endocarditis appears to persist as a consequence of dental treatment.

Since the development of antibiotics, the mortality of endocarditis has decreased; the incidence, however, has not changed.[13] Furthermore, up to 30% of the confirmed cases of endocarditis are attributed to antibiotic resistant microorganisms.[14–16] Thus, when implemented, prophylaxis may not prevent the development of endocarditis.[14] Should therapeutic antibiotic protocols for other systemic conditions increase the frequency of antibiotic-resistant *S. sanguis*, prophylaxis regimens for endocarditis may not be effective.

In the pediatric population, several different antibiotic regimens are currently recommended for the treatment of otitis media. With more than 80% of children having experienced at least one episode of otitis media prior to the age of three, and 46% having more than three episodes,[17] these patients represent a significant potential source of antibiotic resistant organisms. Therefore, we sought to investigate the development of antibiotic resistant oral streptococci following administration of antibiotic therapy for otitis media.

Methods

Subject selection

The experimental group of 25 patients were randomly chosen from a pool of children between the ages of three and six years who had recently completed ten days of antibiotic therapy for otitis media (Park Nicollet Medical Center, Minneapolis, MN) with amoxicillin, Septra® (trimethoprim-sulfamethoxazole), or Pediazole®(erythromycin-sulfisoxazole). A control group of

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Table 1. Characteristics of Subject in Study

Parameters	Otitis Media Patients	Healthy Controls
N	25	25
Males	12	12
Females	13	13
Age (months)	51±12*	53±12*
Weight (lbs)	39±9*	39±10*
Days since last receiving antibiotic prescription	26±10*	>730
Days on last antibiotic course	10	0
Total days on antibiotics in the last 6 months	32±15*	0
MS positive, %**	35±2*	35±2*
S. sanguis, %†	9±2*	9±2

* Mean ± SD. **Percentage of total bacterial counts that grew as colonies on Mitis salivarius selective agar. † Percentage of total bacterial counts that represented *S. sanguis* colonial morphology.

age- and sex-matched children were also clinic patients selected from a pool of healthy children who were scheduled for routine medical examinations and treatments. Children in the control group had received no antibiotics with in the past 24 months. Siblings of children in the experimental group were excluded from the control group to avoid sampling of resistant organisms that may have been transferred within the family unit. Complete medical and antibiotic use history, verified by medical records, and informed written parental consent were obtained prior to sampling dental plaque. The protocol had been reviewed and approved by the Committee on the Use of Human Subjects in Research of the University of Minnesota.

Microorganisms: growth, identification, and preparation

Samples of supragingival dental plaque were collected from the buccal surfaces of both mandibular first primary molars using sterile cotton swabs. Samples were placed into tubes containing 0.5 mL of reduced Todd Hewitt broth (THB) and then plated onto blood and mitis-salivarius agars. The blood agar plates were incubated anaerobically for four days. The mitis-salivarius plates were incubated anaerobically for two days and aerobically for an additional two days. Strains of *S. sanguis* were isolated from MS plates based upon colony morphology[18] and verified by biotyping.[19]

Antibiotic resistance testing

Antibiotic gradient plates[20] were prepared with THB agar and used to precisely determine the level of antibiotic resistance. Agar slants (3°) were prepared in square petri dishes. After cooling to room temperature, the agar slants were overlaid with level agar containing antibiotic. Linear gradients were created in separate dishes from 0 to 25 μg/mL penicillin, 25 μg/mL amoxicillin, 100 μg/mL trimethoprimsulfamethoxazole, and 100 μg/mL erythromycin.

Each isolate of *S. sanguis* was grown overnight (5% CO_2, GasPak, 37°C) in THB to mid-log phase. *S. sanguis* suspensions (0.15-mL containing 10^9 cells) were then spread on the surface of triplicate antibiotic gradient plates using sterile glass spreaders and incubated for 24–48 hours. Minimal inhibitory concentrations (MIC) were determined to be the least concentration of antibiotic that caused complete inhibition of growth.

To facilitate comparisons to data obtained in gradient plates, MICs were also determined by standard tube tests. Dilutions of each antibiotic were prepared in cation-adjusted Mueller-Hinton broth supplemented with 2.5% lysed horse blood with the following antibiotic concentrations: for penicillin and amoxicillin, concentrations were 25, 20, 15, 10, 5, 4, 3, 2, 1, and 0.1 μg/mL; for trimethoprim-sulfamethoxazole and erythromycin, concentrations were 100, 75, 50, 40, 30, 25, 20, 15, 10, and 5 μg/mL. Each set of tubes, which contained 0.9 mL broth, was inoculated with 0.1 mL containing 10^7 cells of one of nine isolates of *S. sanguis*. Control tubes containing each inoculum and broth without antibiotic and negative control tubes containing only broth were included. Following overnight incubation (5% CO_2, GasPak, 37°C) the tubes were examined for growth. The lowest concentration of each antibiotic showing no growth was recorded as the MIC.

Strains were considered to be resistant to a specific antibiotic if the MIC was greater than the average expected blood levels of each antibiotic.[21] This definition is the basis of the National Committee for Clinical Laboratory Standards (NCCLS) guidelines for resistance. The NCCLS values for resistance for each antibiotic are: penicillin, 4 μg/mL; amoxicillin, 4 μgmL; trimetho-primsulfamethoxazole, 4.76 μg/mL; and erythromycin, 8 μg/mL.[22] These same values were used to determine resistance in this study.

Platelet interactions

All strains of *S. sanguis* were tested for interactions with human platelets. The bacteria were grown overnight in THB as described above and washed twice with cold 0.01 M sodium phosphate buffer, pH 7.4 with 0.9% sodium chloride (PBS). The ability of these strains to adhere to washed human platelets was determined using the platelet-bacterial adhesion assay.[23] *S. sanguis* induction of platelet aggregation was studied by aggregometry methods also described earlier.[23]

Statistical management of data

Antibiotic resistance was expressed as MIC. Correlation coefficients were determined for relationships between the patient factors and experimental factors. All data was entered into a database program (Microsoft® Access) and analysis using the Statistical Package for Social Sciences (SPSS).

Results

Patient characteristics

Twenty five patients who had received antibiotics and age- and sex-matched controls were recruited for this study (Table 1). Within the study population, no significant differences were seen between males and females or between children of different ages, except for weight, which increased with age. The children in the experimental group had received antibiotics within an average of 26 days (range=11 to 40 days) of sampling the dental plaque. Including the recent 10-day regimen, these patients received an average of 32 days of antibiotic

Table 2. Antibiotic Resistance of S. sanguis Isolated from Children's Dental Plaque

Variables	Otitis Media Patients	Controls
% strains showing resistance to:		
Any antibiotic	60	0
Penicillin	24	0
Amoxicillin	32	0
Penicillin and amoxicillin	20	0
Trimethoprim-sulfamethoxazole	52	0
Erythromycin	48	0
Two or more unrelated antibiotics	28	0
MIC for all strains tested•	N=29••	N=25
Penicillin	2.6±2.6	0
Amoxicillin	3.2±4.1	0
Trimethoprim-sulfamethoxazole	38±41	0
Erythromycin	8.2±11.1	0
MIC of only the resistant strains•		
Penicillin (N=7)	5.9±2.1	–
Amoxicillin (N=9)	8.8±2.6	–
Trimethoprim-sulfamethoxazole (N=15)	89±10	–
Erythromycin (N=14)	19±5	–

• in μg/mL; mean ± SD •• One strain of *S. sanguis* was isolated in 21 of the subjects. Two strains were isolated in four individuals. Therefore, the total number of strains tested was 29.

therapy within the previous six months, while the control subjects received no antibiotics within 24 months of sampling.

Antibiotic resistance

There was no difference in the frequency of isolation of strains of *S. sanguis* between the otitis media patients and healthy controls (Table 1). At least one strain of *S. sanguis* was isolated in each individual. In four of the experimental subjects, two morphologically distinct strains were isolated and tested. In each of these cases, one strain showed antibiotic resistance and one strain did not.

The percent resistant strains and the mean MIC of all isolates of *S. sanguis* are presented in Table 2. No strain of *S. sanguis* isolated from the control subjects was resistant to any of the antibiotics tested. About 60% of all isolates in the experimental group were resistant to at least one antibiotic. Thirty-two percent of all tested strains from the experimental group of patients were resistant to amoxicillin. Twenty-four percent were resistant to penicillin. Twenty percent of the strains were resistant to penicillin and amoxicillin, whereas 28% were resistent to two or more unrelated antibiotics.

Resistance to penicillin and amoxicillin was inversely related to the age of the child and the length of time since exposure to amoxicillin (Table 3). In contrast, resistance to trimethoprim-sulfamethoxazole and erythromycin showed no correlation with the age of the child or the length of time since exposure to the antibiotic. In fact, the emergence of erythromycin-resistant strains showed no relationship to the antibiotic therapy received (data not shown).

Platelet interactions

Since the emergence of antibiotic resistance in these bacteria would complicate therapy and may alter expression of virulence factors, interactions with human platelets were also analyzed. The patient age, sex, antibiotic therapy received, and level of antibiotic resistance had no effect on the ability of the *S. sanguis* isolates to adhere to or induce aggregation of heterologous human platelets (data not shown).

Discussion

In this study, we demonstrated that about 60% of children in the experimental group did harbor antibiotic-resistant *S. sanguis*. Colonial morphology on blood agar and mitis-salivarius plates, was used to initially identify strains of *S. sanguis*,[18] which were subsequently isolated, biotyped, and evaluated for antibiotic resistance. Therefore, it is possible that our results represent an underestimation of the presence of antibiotic resistant strains of *S. sanguis* present in the dental plaque of children receiving antibiotic therapy for otitis media.

While antibiotic therapy was associated with the isolation of resistant strains, it was not associated with a significant long-term change in the oral flora of these children. In this study, where the otitis media patients were sampled on average 26 days after completion of antibiotic therapy, we found no difference in the frequency of isolation of *S. sanguis* between the otitis media patients and healthy controls. These results are similar to previous studies that have demonstrated an initial reduction in the total streptococcal flora during periods of treatment, with the normal flora re-established within 30 days.[24]

THB agar plates containing a concentration gradient of each antibiotic were used to determine the resistance (percent resistant strains and MIC) of each isolated strain of *S. sanguis*. Antibiotic gradients in agar were used to avoid the disadvantages of other techniques. In serial dilution methods, concentrations of the antibiotic increase stepwise. In doing so, the accuracy of a continuous gradient is lost. While paper-disk diffusion techniques provide continuous antibiotic gradients, the steep logarithmic function offers poor discrimination for quantitation. The antibiotic gradient plates are designed to provide a gradual proportional increase in the antibiotic concentration and an accurate determination of the level of antibiotic resistance. To demonstrate the usefulness of the antibiotic gradient technique and to facilitate comparisons to methods used in clinical laboratories, standard tube dilution tests were performed. MICs measured using gradients plates were 1.2 to 1.3 times greater than in standard broth dilution

Table 3. Correlation Coefficents for Antibiotic Resistance

Antibiotic	Patient Age	Time since exposure
Penicillin	-0.5959•	-0.8120•
Amoxicillin	-0.6654•	-0.8830•
Trimethoprim-sulfamethoxazole	0.2745	-0.0967
Erythromycin	0.0075	-0.1964

• $P<0.001$

tests (data not shown). Similar results were reported when the mitis-salivarius agar dilution test was compared with standard tube dilutions.[25]

Oral microorganisms, including streptococci, which are resistant to antibiotics, have been recovered in gingival crevicular fluid, saliva, and subgingival and supragingival plaques. Cross-sectional surveys involving adult volunteers suggest that from 2 to 25% of adults may harbor microorganisms resistant to at least one antibiotic.[26–29] Furthermore, in one study conducted in the Netherlands, researchers found that about 11% of cases of viridans streptococcal endocarditis were due to resistant strains.[16]

Studies of children have found that 78-81% of rheumatic children on oral penicillin prophylaxis harbored penicillin-resistant streptococci.[30–32] Furthermore, streptococci specifically resistant to penicillin were identified in 35% of nasopharyngeal cultures obtained from healthy children during a one-year period of sampling.[33] Our results demonstrated that antibiotic resistant strains of oral streptococci may emerge more frequently in children than previously reported in adults.[26–29] The frequency of resistance decreased with the time since cessation of therapy and the age of the child, similar to data reported earlier by Sprunt, et al.[31] In our study, the pediatric patients we sampled had completed their antibiotic therapy an average of 16 days before plaque sampling. Since the frequency of resistance decreases with time, our data may actually underestimate the level of resistance present immediately following antibiotic therapy.

Antibiotic resistance is a growing problem in medicine and dentistry, especially resistance to multiple drugs.[34–36] Resistance to pharmacologically dissimilar antibiotics has been previously shown to occur in dental plaque bacteria.[26, 27, 29, 35] In our study of children, a large percentage (48%) of all *S. sanguis* isolated from the experimental group were resistant to more than one antibiotic. While penicillin and amoxicillin resistant strains emerged and persisted, more than one-fourth of the strains tested were resistant to two or more unrelated antibiotics. Our data confirm that resistance to pharmacologically different antibiotics can develop and show that antibiotic administration may be a factor since control (untreated) subjects showed no resistance.

Several interrelated factors have contributed to the emergence and spread of multiply-resistant organisms.[36] First, mutations can occur in common resistance genes that will extend the spectrum of resistance of an organism. Second, bacteria can exchange genetic information by transformation, transduction, or conjugation with other bacteria, thereby transferring well-known genes into new hosts. And finally, selective environmental pressures can encourage or enhance the proliferation of resistant strains.

The establishment of singly resistant or multiply resistant strains of *S. sanguis* within the oral cavity could impact therapy for systemic infections such as bacterial endocarditis. Because the interactions of *S. sanguis* with human platelets in vitro were not affected by the antibiotic resistance of the isolates, the thrombogenic potential of *S. sanguis* bacteremia[37–38] is probably unaffected. Therefore, the emergence of antibiotic resistant *S. sanguis*, with no apparent change in thrombogenicity, may accompany therapeutic antibiotic use in pediatric patients and complicate the prophylaxis and therapy for bacterial endocarditis. If a series of dental appointments is required, or if the patient is receiving antibiotic therapy for a medical condition such as otitis media, the American Heart Association suggests that the dental practitioner observe an interval of time between procedures to both reduce the potential for emergence of resistant organisms and allow repopulation of the mouth with antibiotic-susceptible flora.[12] If treatment needs make postponement unfeasible, antibiotic prophylaxis in combination with an antiseptic mouth rinse applied immediately prior to dental procedures may reduce the incidence and magnitude of bacteremia.[39]

Conclusions

Based upon the research presented, we conclude that:

1. Children receiving antibiotic treatment for otitis media harbor resistant oral streptococci.
2. Resistance to penicillin and amoxicillin decreased with the age of the child and the length of time since exposure to the antibiotic.
3. Caution must be excercised when treating child patients at risk for endocarditis, especially when the child is receiving therapeutic antibiotics for medical and/or dental infections.

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