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Bacterial interference in nasopharyngeal bacterial flora of otitis-prone and non-otitis-prone children

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Abstract. *Bacterial interference in nasopharyngeal bacterial flora of otitis-prone and non-otitis-prone children.* The quantitative bacteriology of the adenoid was studied in 34 otitis-prone and 25 non-otitis prone children. Viridans *streptococci* appeared to be the predominant normal flora in children who are non-otitis prone. There was a significant decrease in viridans *streptococci* in the otitis-prone child compared to the non-otitis-prone child. There was a significant increase in nontypable *Haemophilus influenzae* (NTHI) in the otitis-prone child. The mechanisms responsible for this alteration of the micro-ecology of bacteria of the nasopharynx may be related, in part, to bacterial interference or to the inappropriate use and over-use of antibiotics. *In vitro* inhibition of growth of NTHI was demonstrated with selective strains of viridans *streptococci.* A preliminary analysis of an inhibitory strain and a non-inhibitory strain of viridans *streptococci* are presented and their biochemical profiles and antibiotic sensitivities were entirely different. A possible mechanism for the inhibition on NTHI by viridans *streptococci* has been suggested. This mechanism may be related to an alteration of pH in the growth media or the possibility of the utilization of nutrients required for growth of NTHI.

Introduction

The events associated with altered homeostasis of the nasopharynx and middle ear cleft that lead to otitis media (OM) and otitis media with effusion (OME) with NTHI are not completely known. We have previously reported on the micro-ecology of the nasopharyngeal bacterial flora in otitis-prone and non-otitis-prone children (1). Quantitative bacteriology of the adenoid demonstrated a definite inverse relationship between α-hemolytic *streptococci* (viridans *streptococci*) and NTHI in non-otitis-prone children and otitis-prone children. The factors responsible for this alteration of normal flora remain to be elucidated. However, the association of a viral upper respiratory tract infection appears to be commonly associated with increased colonization of the nasopharynx and perhaps with increased adherence of NTHI to the nasopharyngeal mucosa (6, 7). Since identical strains of NTHI in the nasopharynx and the middle ear occur at the time of otitis media (2), it is reasonable to assume that prevention of colonization with NTHI or minimizing colonization in the nasopharynx may prevent the development of OM and sinusitis with that organism.

Bacterial interference, the process when one bacterial organism prevents colonization with a second organism (3), may be one method to protect the nasopharynx from colonization

with pathogens. Mechanisms of bacterial interference include bacteriocins (bacterial polypeptides which prevent growth of another organism), nutritional competition, and modification or masking of tissue receptors (10). Bacterial interference may be homologous or heterologous (3, 10, 12, 13). For example, viridans *streptococci* can prevent colonization with Streptococcus pneumonia, beta-hemolytic Streptococcus and Staphylococcus aureus (8, 13, 16).

The aims of the present study were to determine the quantitative nasopharyngeal bacteriology of the viridans *streptococci* and NTHI, an organism which has become the predominant bacterial species associated with both acute suppurative OM, and more chronic persistent effusion of the middle ear called otitis media with effusion (OME) (4). The results of this study suggest that there is a micro-ecological relationship between viridans *streptococci* and NTHI. Quantitative bacteriological studies in the otitis-prone and the non-otitis-prone child suggest an inverse relationship between the presence of viridans *streptococci* and NTHI in the nasopharyngeal bacterial flora. We report for the first time the ability of viridans *streptococci* to inhibit the growth of NTHI in an *in vitro* culture system. Finally, characterization of an inhibitory strain and the possible mechanism of inhibition are reported.

Materials and methods

Study population

Fifty-nine children were studied. 34 were defined as otitis-prone by having had at least six episodes of acute suppurative OM in the first two years of life or four episodes in the first year of life ; 25 children were considered non-otitis-prone, as defined by having less than three episodes in the first two years of life.

The 34 otitis-prone children underwent adenoidectomy and bilateral or unilateral tympanostomy with tubes because of recurrent OM or persistent middle ear effusion. Adenoidectomy was performed in 25 non-otitis-pronechildren because of nasal obstruction. The demographics of the study are tabulated in Table I.

Bacteriology

Upon receipt in the laboratory, the adenoid tissue was weighed, homogenized, and inoculated onto sheep blood agar, chocolate agar, MacConkey agar and phenylethyl alcohol agar (PEA) using 1 µl and 10 µl calibrated loops. The blood, PEA, and chocolate agar plates were incubated at 37°C with 2-5% CO_2 and identified by conventional bacteriologic means. The numbers of colonies of bacteria per gram of adenoidal tissue were determined for all organisms.

Table I

Demographics of the study

Variable	Otitis-prone n = 34 Mean (S.D.)	Non-otitis prone n = 25 Mean (S.D.)	p value
Age (months)	40.9 (17.1)	50.4 (18.5)	.048
Sex			
Male	17	18	.153
Female	17	7	
Fluid			
Yes	28	6	.001
No	6	19	
Antibiotics			
Yes	22	8	.027
No	12	17	
Duration of antibiotic	1.8 (2.6)	.5 (1.0)	.009

Speciation of viridans streptococci *using the Vitek system*

Strains of viridans *streptococci* were identified utilizing the Vitek "automated system" (SH System #0751) (Hazalwood, MO 63042).

Agar overlay technique

This technique was devised for screening viridans *streptococci* (VS) for inhibitory activity against NTHI isolated from the nasopharynx. A standardized suspension (0.5 McFarland, 10^8 cfu/ml) of VS was inoculated over the entire surface of a blood agar plate (5% sheep blood) and incubated overnight at 37°C in 2-5% CO_2. The incubated blood-agar plate was then removed from the incubator and was inspected for uniform distribution of the VS colonies. The plate was then overlaid with 8 ml of freshly prepared *Haemophilus* test media which is a clear media (National Committee for Clinical Laboratory Standards M2-A4, 1992) (M100-S3). The overlaid plate was allowed to solidify. The surface of the overlaid plate was then divided into 8 sections and 8 different strains of NTHI were inoculated onto each section using an inoculating loop. The plates were then incubated overnight at 37°C in 2-5% CO_2. The following day the plates were inspected for inhibition of the growth of NTHI in each section. The scores were as follows : no growth = total growth inhibition of NTHI by VS ; growth = no inhibition of NTHI by VS ; partial growth = partial inhibition of NTHI by VS. A total of 21 VS was tested against 14 strains of NTHI.

Photography

Using TMY 400 ISO BHW 35 mm film (Kodak Co., Rochester, NY), and a Micro-Nikkor 50 mm-macro and one 200 watt studio strobe flash with the center point of light focused on the center point of the agar plate, and the plates angled at approximately 10° away from the lens, pictures were taken of the agar overlay technique to demonstrate inhibition of bacterial growth of NTHI.

Characterization of viridans streptococci *(P), an inhibitor strain and viridans* streptococci *(B), a non-inhibitor strain*

Two strains of viridans *streptococci* were characterized by their biochemical characteristics and antibiotic sensitivities, as well as by their ability to inhibit the *in vitro* growth of NTHI on chocolate agar. The Rap ID STR System (Innovative Diagnostics, Atlanta, GA) was used to identify these two strains of viridans *streptococci*. Furthermore, antibiotic sensitivity to gentamicin, penicillin, ampicillin, tetracycline, and trimethropin/sulfa (TS) were also used for comparison of these two organisms.

Co-cultivation of viridans *streptococci* (P) and viridans *streptococci* (B) were performed in HTM (*Haemophilus* test media) broth for 18 hours with a strain of NTHI. The broth was then gram stained and plated on chocolate agar. In addition, the pH of the broth in which each strain of viridans *streptococci* was grown was also measured. In addition, co-cultivation of the organisms were performed on solid chocolate agar and the growth adjacent to viridans *streptococci* was observed.

Statistics

All data obtained in the study, including demographics and colonies per gram of adenoid, percentage of organisms in the nasopharyngeal tonsil, were analyzed using the Statistical Package for the Social Sciences (SPSS-PC). Comparisons between the two groups were made using the two-tailed t-test (continuous data), and the Chi square (discrete data). In addition, non-parametric studies were calculated using the Mann-Whitney (M-W) for continuous data which was not normally distributed. All of the variables in this study were evaluated for both skewness and normality. The t-test was used only if the data was normally distributed and the M-W was used otherwise. Informed consent was obtained from each participant at the time of entry into the study.

Results

Table I reviews the demographics of the children in the study. The non-otitis-prone children were significantly older than the otitis-prone children. There was a significant difference in the number of children in the otitis-prone group who had received antibiotics compared to the non-otitis-prone group. The duration of antibiotics was also significantly greater for the otitis-prone children as compared to the children in the non-otitis-prone group. Most of the children in the otitis-prone group were on cephalosporins, or amoxicillin with claviculinic acid. The duration of antibiotics in weeks is related to the number of weeks prior to the surgery; that is, the children who had been on antibiotics were on antibiotics right up until the time of surgery. The duration of the antibiotics, and its relation to the time of adenoidectomy is critical for the interpretation of the results. It is also emphasized that the children who were otitis-prone continued to have recurrent OM up until the time of surgery, although the definition used in this study, as mentioned previously, was related to the number of episodes in the first two years of life.

Table II summarizes the data for the quantitative bacteriology in the adenoids between the otitis-prone group and in the non-otitis-prone group. A significantly greater number of colonies of viridans *streptococci* occurred in the non-otitis-prone group compared to the otitis-prone group ($p = .025$). In contrast to this, the number of colonies of NTHI were significantly greater than the number of colonies of this organism in the non-otitis-prone group ($p = .01$). In general, there was a trend for more colonies per gram of *S. pneumoniae* in the otitis-prone group, but this did not reach statistical significance between the groups. *Moraxella catarrhalis* was not significantly different between the groups. Finally, the total number of organisms in the adenoidal tissue in both groups was not significantly different.

Table III shows the percentage of bacterial flora in otitis-prone and non-otitis-prone groups. A significantly greater percentage of -viridans *streptococci* occur in the non-otitis-prone group ($p = .002$). In contrast, the percentage of NTHI was greater in the otitis-prone group ($p = .01$). There was not a significant difference in the group for *S. pneumoniae* and *Moraxella catarrhalis*.

Two species of viridans *streptococci*, mitis and sanguis, were the two predominant species in both groups and there was no significant difference between the two groups of children (Table IV).

Finally, Table V summarizes the ratio between viridans *streptococci* and NTHI in the two groups. The mean ratio of viridans *streptococci* to NTHI (colonies/gram) was significantly greater in the non-otitis-prone group as compared to the otitis-prone group, and finally the ratio between the percentage of -viridans *Strep* and NTHI was again significantly greater for the non-otitis-prone group.

Figure 1 shows a bacterial overlay technique as it relates to NTHI and viridans *streptococci*. This viridans *streptococcus* organism was able to inhibit all strains of NTHI. Only one of 21 strains inhibited all strains of NTHI with which it was incubated. Eleven strains had intermediate activity (Fig. 2), and 9 strains had no inhibitory activity whatsoever (Fig. 3). A summary of the overlay technique in 202 *in vitro* cultures is summarized in Fig. 4.

Table VI summarizes the identification and characteristics of the inhibitor strain viridans *streptococci* (P) and the non-inhibitor strain, viridans *streptococci* (B). The inhibitor strain was Inulin negative and NAG (p-nitrophenyl β D-N-acetyl glucosaminide) positive, whereas the non-inhibitor strain was Inulin positive and NAG negative. The antibiotic susceptibilities were significantly different in that the inhibitor strain had an mic of ≤ 0.03 μg/ml to penicillin, but the non-inhibitor strain had an mic of 2 μg/ml to penicillin. Sensitivities to tetracycline, trimethoprin-sulfamethoxazole and gentamicin, as well as ampicillin, were all

Table II

Quantitative bacteriology of the adenoid
in otitis-prone and non-otitis-prone children

Species	Otitis-prone n = 34 Mean (S.D.)	Non-otitis prone n = 25 Mean (S.D.)	p value
Viridans *Streptococci*	3.20 (3.01)*	7.66 (7.80)	.025
Nontypable *Haemophilus* influenzae	6.73 (9.52)	1.53 (3.27)	.010
Streptococci pneumoniae	1.34 (3.07)	0.93 (2.76)	.305
Moraxella catarrhalis	1.54 (3.66)	1.01 (2.79)	.909
Other	6.09 (8.41)	4.95 (6.29)	.824
Total	17.99 (13.44)	16.27 (10.44)	.924

* = number of colonies bacteria $\times 10^4$/gm of adenoid.

Table III

Percentage of bacterial flora in otitis-prone
and non-otitis-prone children

Species	Otitis-prone n = 34 Mean (S.D.)	Non-otitis prone n = 25 Mean (S.D.)	p value
Viridans *Streptococci*	22.6 (23.1)	47.8 (33.6)	.01
Nontypable *Haemophilus* influenzae	27.2 (30.4)	7.8 (17.6)	.01
Streptococci pneumoniae	8.4 (17.8)	5.5 (15.4)	.28
Moraxella catarrhalis	6.3 (17.9)	6.0 (16.9)	.99
Other	22.1 (25.6)	30.4 (30.8)	.29

Table IV

Percentage of Viridans *Streptococci* (VS) species
in otitis-prone and non-otitis-prone children

Species	Otitis-prone n = 34 Mean (S.D.)	Non-otitis prone n = 25 Mean (S.D.)	p value
Mitis	32.6 (23.2)	45.4 (35.3)	.193
Sanguis	19.8 (19.1)	25.0 (27.2)	.493

Fig. 1
Agar overlay technique devised for screening viridans *streptococci* (VS) or inhibitory activity against NTHI. The entire surface of the blood agar plate (5% sheep blood) was incubated with viridans *streptococci* which shows as a diffuse white layer underlying the transparent *Haemophilus influenzae* agar. The overlaid agar is transparent and has been streaked with eight separate strains of NTHI. In this figure there is complete inhibition of the growth of *Haemophilus influenzae*.

Fig. 2
Agar overlay technique using viridans *streptococci* in the lower blood agar plate and the agar on the top is streaked with eight different strains of NTHI which show as small black dots. This species of viridans *Strep* was capable of inhibiting three of the eight strains.

Fig. 3
Agar overlay technique using viridans *streptococci* in the bottom layer of blood agar. Eight separate strains of NTHI are shown in the overlaid transparent agar. This viridans *streptococci* strain was capable of inhibiting only one strain of NTHI.

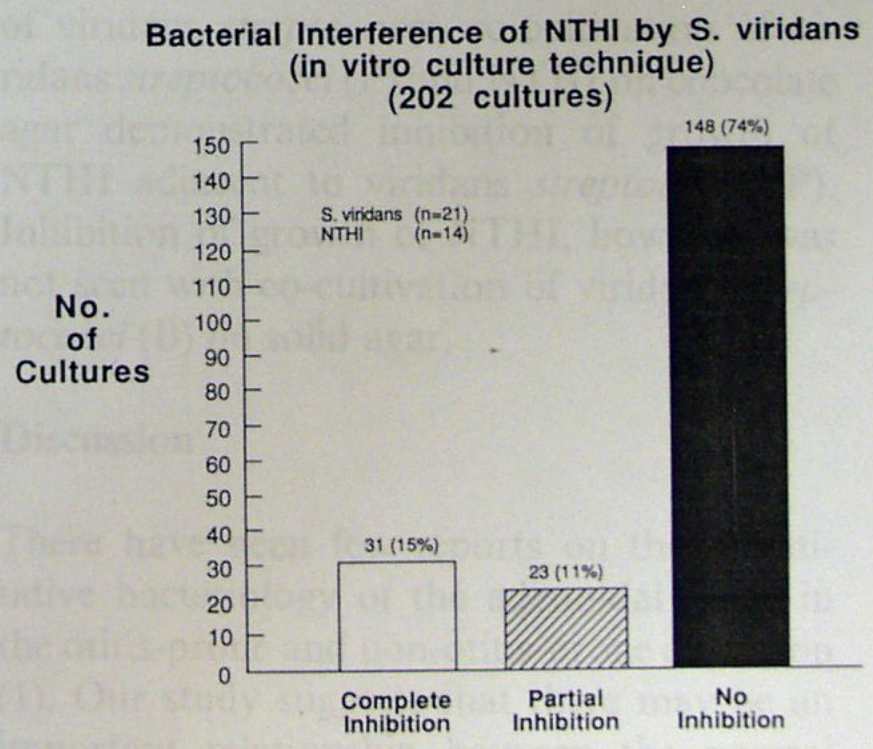

Fig. 4
Bacterial interference on nontypable *Haemophilus influenzae* by *streptococcus* viridans. Fifteen percent of the cultures of nontypable *Haemophilus influenzae* were inhibited by *streptococcus* viridans. Eleven percent showed partial inhibition by viridans *streptococci* and 74% showed no inhibition.

Table V

Ratio of Viridans *Streptococci* (VS)
to nontypable *Haemophilus* influenzae (NTHI)
in otitis-prone and non-otitis-prone children

Species	Otitis-prone n = 34 Mean (S.D.)	Non-otitis prone n = 25 Mean (S.D.)	p value
Ratio of VS/NTHI (col./gm)	103.9 (208.1)	453.6 (706.8)	.001
Ratio of VS/NTHI (%)	13.4 (23.8)	35.0 (36.2)	.001

Table VI

Comparison of inhibitor strain of Viridans *streptococcus* (P)
and Non-inhibitor Viridans *streptococcus* (B)

	1 Inulin	2 NAG	3 Gent	4 T/S	5 PEN	6 AMP	7 Tet
V.S. (P) (inhibitor strain)	—	+	6	S	S	S	R
V.S. (B) (non-inhibitor strain)	+	—	≤ 1	R	R	R	S

1 formation of acidic product from carbohydrate utilization.
2 hydrolysis of p-nitrophyl ^{B}, D-N-acetyl glucosamide.
3 gentamicin.
4 trimethoprin/sulfa.
5 penicillin.
6 ampicillin.
7 tetracycline.

different. These strains, however, were both *Streptococcus sanguis* II.

When viridans *streptococci* (P) was co-cultivated with NTHI in HTM (*Haemophilus* test medium) broth for 18 hours and then subcultured on chocolate agar, only the viridans *streptococci* grew ; NTHI did not grow at all. When SV was grown in BHI broth, the pH of the broth was measured between 4.5 and 5.0. Normally this broth has a pH of 7.2.

After growing the inhibitor and non-inhibitor strains of viridans *streptococci* in BHI broth for 18 hours at 36°C, the sterile filtrate from each strain was used to saturate filter paper disks. The disks were placed on the surface of a chocolate agar plate uniformly streaked with a 0.5 McFarland suspension of NTHI. The plates were incubated in 2.5% CO_2 at 36°C for 18 hours. No zone of inhibition was produced around either of the filtrate saturated disks.

In contrast to the findings with the filtrates of viridans *streptococci*, co-cultivation of viridans *streptococci* (P) and NTHI on chocolate agar demonstrated inhibition of growth of NTHI adjacent to viridans *streptococci* (P). Inhibition of growth of NTHI, however, was not seen with co-cultivation of viridans *streptococci* (B) on solid agar.

Discussion

There have been few reports on the quantitative bacteriology of the adenoidal tissue in the otitis-prone and non-otitis-prone condition (1). Our study suggests that there may be an important relationship between the normal flora, which consists mainly of the viridans group of *streptococci* and NTHI that frequently co-exist in the adenoidal tissue with viridans *streptococci*. This study indicates that a significantly greater number of colonies of viridans *streptococci* are present in the ade-

noids of non-otitis-prone children as compared to the otitis-prone group of children. In contrast, a significantly higher number of NTHI is present in the otitis-prone group as compared to the non-otitis-prone group. The long-term use of prophylactic as well as therapeutic antibiotics could lead to decrease in the colonization of viridans *streptococci* and possibly remove an important normal mechanism for the prevention of colonization and replication of potential pathogens, such as NTHI and *Streptococcus pneumoniae*.

There is abundant evidence in the literature over the last 30 years that an interrelationship exists among the so-called indigenous bacterial flora of the pharynx and nasopharynx and potential pathogens, such as *Staphylococcus aureus, Streptococcus pneumonia*, and β-hemolytic *streptococci*. It is possible that indigenous normal flora may inhibit colonization of the nasopharynx with such potential pathogens. Viridans *streptococci* appear to be capable of inhibiting colonization and replication of a variety of pathogenic organisms, including *S. pneumoniae*, β-hemolytic *Streptococcus*, and *S. aureus* (8, 13, 16). The mechanisms by which the normal flora inhibit colonization may be related to the production of high molecular protein antibiotics by resident viridans *streptococci*, or may be due to utilization of nutrients in the nasopharyngeal environment which are then not available for potential pathogens. The development of *candidiasis* after suppression of the resident flora by broad spectrum antibiotics suggests that the inhibitory mechanisms of normal flora are also active against fungal as well as bacterial pathogens (9). We present evidence for the first time that a similar relationship may exist between the viridans *streptococci* and NTHI. *In vitro* inhibition of the growth of NTHI by certain strains of viridans *streptococci* has been shown. Theoretically, such strains of viridans *streptococci* may be able to inhibit *in vivo* colonization of NTHI.

The inhibitor strain, viridans *streptococci* (P) and the non-inhibitor strain, viridans *streptococci* (B) were both *Streptococcus sanguis II* species. However, they were significantly different in some of their biochemical characteristics as well as their antibiotic sensitivities. The filtrate of the inhibitor strain however, does not appear to possess any factor which can inhibit the growth of NTHI.

It is more likely that co-culturing of the inhibitor strain of viridans *streptococci* with NTHI in culture medium changes the pH of the medium, or utilizes nutrients in the medium which are than not available for NTHI. In summary, the mechanism of bacterial interference between viridans *streptococci* and NTHI, may be similar to the mechanism described by the Sanders group concerning the mechanism of inhibition of Group A *streptococci* by viridans *streptococci* (15).

A number of investigators have shown that competitive adherence is a mechanism of bacterial interference. That is, the bacteria that first adhere to nasopharyngeal cells interfere with the colonization of subsequent bacteria (14, 17). Thus, if viridans *streptococci* adhere to the nasopharyngeal mucus or nasopharyngeal epithelial cells first, bacterial organisms responsible for acute inflammation in otitis media might be prevented from colonizing into the nasopharyngeal mucus or epithelial cells.

Treatment of OM has been directed primarily at the eradication of pathogenic bacteria with appropriate antibiotics. However, more than 40 years after the introduction of chemotherapeutic agents to destroy bacteria, clinical otolaryngologists are over-whelmed with the numbers of young patients with recurrent acute and persistent OM, and with increasing incidence of bacterial resistance to antibiotics. It is not surprising that in the last decade great interest has been focused on other approaches to the treatment of OM, such as the development of vaccines directed against outer membrane proteins or capsules of some of these organisms.

It is conceivable that a new approach to the treatment of OM may be to maintain a normal bacterial flora in the nasopharynx. Thus, attempts to build a normal flora have

intrigued clinicians for over 100 years, and there is the prospect of increasing resistance to infection by supplementing the normal flora with non-pathogenic bacteria that have special capabilities to inhibit pathogenic microorganisms. In this regard, Roos (11) and collaborators have treated repeated β-hemolytic *Streptococcal* tonsillitis by recolonization with viridans *streptococci*. The results of that report, although based on a small clinical study, indicated that recolonization with interfering viridans *streptococci* restored the normal defense mechanisms and hindered recurrent infection in several families with recurrent tonsillitis by Group A *streptococci*. Certainly one of the therapeutic microbes might be the viridans *streptococci*, particularly *streptococci mitis, sanguis*, or *salivarius*. Whether or not maintaining a normal flora in the nasopharynx or adenoid would be beneficial to the otitis-prone child deserves further investigation. Since we have now entered into the age of genetic engineering and recombinant DNA, perhaps a microorganism can be designed to meet these stipulations if a naturally occurring one cannot be obtained. When based on sound ecological principles, bacteriotherapy and bacterioprophylaxis seem appropriate and efficient means of resisting infectious microorganisms at the portals of entry into the body (5).

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