INFECTION AND IMMUNITY, Sept. 1971, p. 264-268

Vol. 4, No. 3
Printed in U.S.A.

Ability of *Veillonella* and *Neisseria* Species to Attach to Oral Surfaces and Their Proportions Present Indigenously

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Received for publication 7 April 1971

The present study describes the distribution of *Veillonella* and *Neisseria* species in the human oral cavity and indicates that their ability to attach to oral surfaces correlates with their proportions found in various sites of the mouth. The mean percentages of *Veillonella* and *Neisseria* of the total flora cultivable on anaerobic blood-agar plates was found to be: plaque, 0.75 and <0.13, respectively; lip, 0.38 and <0.05; cheek, 0.66 and <0.14; tongue dorsum, 9.4 and <0.12; saliva, 5.0 and <0.9. The ability of *Veillonella* and *Neisseria* species to attach to tooth surfaces was studied by cleaning the labial surfaces of incisors to render them relatively free of viable bacteria. Samples taken 1 hr later contained <0.27% *Veillonella* and <0.4% *Neisseria*, whereas saliva to which these teeth were exposed contained 20-fold higher proportions of *Veillonella*. These data indicate that *Veillonella* and *Neisseria* species possess a feeble ability to attach to cleaned teeth. The ability of these organisms to adhere to other oral surfaces was determined by introducing mixtures of streptomycin-resistant strains into the mouths of volunteers for 5 min. Labeled strains of *Streptococcus sanguis* and *S. salivarius* were included for comparative purposes. Analysis of samples obtained from oral surfaces after 45 min indicated that *Veillonella* and *Neisseria* adhere very poorly to preformed dental plaque as compared to *S. sanguis*. In contrast, *Veillonella* adhered to the tongue dorsum markedly better than *Neisseria*, *S. sanguis*, and *S. salivarius*. The greater ability of *Veillonella* to adhere to the tongue in relation to the other organisms studied correlates with the high proportions of *Veillonella* found on this site. The feeble ability of *Neisseria* to attach to surfaces in the oral cavity is reflected by their low proportions found on these surfaces.

The proportional distribution of many microorganisms varies widely from site to site within the oral cavity (1, 3, 6, 9). This selective bacterial localization in the mouth has been most studied with *Streptococcus* species. It has recently been found that the ability of these organisms to attach to different oral surfaces correlates with their proportions found naturally on these surfaces in the mouth. Consequently, it has been proposed that the selective nature of bacterial adherence may be a major ecological determinant governing the colonization of streptococci in various sites in the mouth (5, 15; van Houte, Gibbons, and Pulkkinen, *unpublished data*). The present investigation describes the proportional distribution of *Veillonella* and *Neisseria* species in different sites in the mouth and indicates that the ability of these gram-negative organisms to attach to oral surfaces also correlates with their proportions found indigenously.

MATERIALS AND METHODS

Subjects and sampling procedures. Twelve females and four males between the ages of 19 and 38 volunteered for this study. No restrictions were placed on their habits or diets except for modifications in oral hygiene as noted. Oral surfaces were rinsed with 5 ml of sterile saline prior to sampling. Epithelial surfaces and precleaned teeth were sampled by vigorous swabbing with Calgiswabs (Colab). Saliva was collected in sterile tubes. Samples of dental plaque were collected from the facial and lingual surfaces of anterior and posterior teeth by using sterile curettes. Care was taken to avoid touching gingival tissue when sampling teeth.

Cultural methods. Calgiswab samples from teeth and soft tissue surfaces were dissolved in 2 ml of sterile Ringer solution containing 1% hexametaphosphate for 1 to 2 min to release the bacteria and were then agitated by using a Vortex mixer for 30 to 45 sec. Appropriate dilutions were prepared in 0.05% yeast extract. Samples of the dilutions were spread by bent glass rods on the surfaces of duplicate plates of

medium. Samples of dental plaque were dispersed in 2 ml of sterile Ringer solution by 10 sec of exposure to sonic oscillation with an MSE sonic oscillator (Measuring and Scientific Equipment, Ltd., London) at an amplitude of 6 μ prior to diluting and plating.

Veillonella were enumerated on the modified medium of Rogosa et al. (14) and identified on the basis of colonial morphology and Gram-stained smears. *Neisseria* were cultured on Trypticase soy agar (BBL) containing 1.9 μg of vancomycin per ml (12). *Neisseria* were identified on the basis of colonial morphology, Gram-stained smears, and the oxidase test. Preliminary experiments indicated that the numbers of *Neisseria* colonies recovered from the Trypticase soy-vancomycin-selective medium were similar to those obtained from Trypticase soy agar alone, which was not selective. All samples were also plated on Heart Infusion-agar plates (Difco) containing 10% horse blood to determine the total cultivable colony count for reference purposes. Plates for the enumeration of *Veillonella* and the blood-agar plates for total cultivable counts were incubated anaerobically in Brewer jars containing an atmosphere of 10% H_2, 10% CO_2, and 80% N_2. Plates for isolation of *Neisseria* species were incubated in an atmosphere of 90% air and 10% CO_2. All plates were incubated at 35 C for 3 days.

Bacteria. *Veillonella* strain C-R and *Neisseria* strain D-R were isolated from participating subjects. They represented the most common colonial types of each organism encountered. *S. salivarius* strain 9GS2 and *S. sanguis* strains MI and H7P were obtained from the culture collection of the Forsyth Dental Center. All organisms were labeled by making them resistant to 2,000 μg of streptomycin per ml by repeated transfer in Trypticase soy broth (BBL) containing increasing concentrations of streptomycin sulfate. The streptomycin-resistant mutants were not found to differ from parent strains in respect to colonial morphology or biochemical characteristics. Periodic examination of streptomycin-labeled strains was made to assure homogeneity of resistance by comparing total viable counts on media with and without 200 μg of streptomycin per ml. Stock cultures were stored aerobically on Trypticase soy agar slants at 4 C and transferred monthly.

Suspensions of streptomycin-labeled *Veillonella*, *Neisseria*, *S. salivarius*, and *S. sanguis* were prepared from overnight Trypticase soy broth cultures. The organisms were washed once and suspended in 0.067 M phosphate buffer (*p*H 6). Suspensions of each organism were adjusted to an optical density of 1.0 at 550 nm and then concentrated 10-fold by centrifugation. Equal volumes of the four cell suspensions were then thoroughly mixed, and the proportions of each organism in the mixture were determined by cultural techniques. These mixtures contained between 5×10^8 and 5×10^9 colony-forming units of each organism per ml.

Proportional distribution of Veillonella and Neisseria species in various sites in the oral cavity. To determine the natural distribution of *Veillonella* and *Neisseria* species in the mouth, samples were obtained from the dorsum of the tongue, the lip, the cheek, saliva, and preformed dental plaque of 10 individuals. *Veillonella* and *Neisseria* species were enumerated as described above, and the data are expressed as a proportion of the total cultivable count.

Ability of natural Veillonella and Neisseria species to adhere to clean tooth surfaces. The ability of *Veillonella* and *Neisseria* species naturally present in the mouth to adhere to the labial and lingual surfaces of the upper cleaned anterior teeth was determined. These teeth were cleaned by using sterile brushes, cups, and pumice. This was followed by five successive swabbings with sterile Calgiswabs to reduce their bacterial populations to negligible levels (less than 100 viable organisms per swab). The subjects then resumed normal activity but refrained from oral hygiene procedures for 1 or 24 hr. Samples of saliva were collected after these periods, and the previously cleaned tooth surfaces were rinsed with sterile saline and sampled for adherent organisms.

Adherence of streptomycin-labeled Veillonella and Neisseria species to preformed dental plaque and the tongue and cheek. Subjects were asked to refrain from oral hygiene procedures for 24 to 48 hr prior to the experiment to allow microbial plaque deposits to accumulate on the teeth. Samples of the washed streptomycin-labeled bacterial mixture (0.4 ml) were placed into the mouths of volunteers who were instructed to distribute it within their oral cavities. The mixture was expectorated after 5 min, and, after 45 min, the plaque and epithelial surfaces were rinsed with sterile saline. Samples of dental plaque and swabbings from the tongue and cheek were collected and cultured for the proportions of each labeled organism. Streptomycin-labeled *Veillonella* and *Neisseria* species were determined on the selective media described previously supplemented with 200 μg of streptomycin per ml. Labeled strains of *S. salivarius* and *S. sanguis* were cultured on plates of Mitis Salivarius Agar (Difco) containing 200 μg of streptomycin per ml. These organisms were distinguished on the basis of their characteristic colonial morphology (2, 15).

RESULTS

The natural distribution of *Veillonella* and *Neisseria* species in various sites in the oral cavities of the 10 subjects studied is listed in Table 1 and 2. These data are expressed as a percentage of the total cultivable flora in each sample. Considerable variation was observed in samples between different subjects, and, consequently, both geometric and arithmetic means were calculated. The geometric means are less influenced by extremes in distribution and thus are more likely to give a better representation. The mean proportions of *Neisseria* were less than 1% of the cultivable flora in all sites studied, whereas *Veillonella* species comprised 5 and 8% of the cultivable organisms present in saliva and on the tongue surfaces, respectively. In other sites studied, *Veillonella* made up less than 1% of the cultivable flora (Tables 1 and 2).

TABLE 1. *Proportions of Veillonella and Neisseria species of the total flora cultivable from various sites in the oral cavities of 10 subjects*[a]

Subject	Per cent cultivable from lip		Per cent cultivable from cheek		Per cent cultivable from tongue		Per cent cultivable from plaque	
	Veillonella	*Neisseria*	*Veillonella*	*Neisseria*	*Veillonella*	*Neisseria*	*Veillonella*	*Neisseria*
1	0.26	0.2	2.5	0.001	15.0	<0.00008	0.6	3.6
2	0.19	0.003	0.06	<0.0009	14.2	0.003	0.9	0.3
3	0.95	1.0	0.01	0.004	4.5	3.5	1.7	0.008
4	1.9	0.16	0.5	0.9	12.5	3.2	0.3	2.0
5	0.03	0.15	0.1	1.3	21.5	1.8	1.3	<0.0001
6	7.2	<0.02	0.2	1.7	2.8	5.5	1.0	0.7
7	0.2	0.009	0.005	0.3	0.3	0.4	<0.2	0.2
8	0.2	<0.0008	7.2	0.8	23.7	5.8	0.3	7.8
9	0.4	0.08	2.8	5.8	23.3	0.005	1.0	0.2
10	1.2	0.5	2.6	<0.2	15.1	0.0006	2.7	0.003
Arithmetic mean	1.2	<0.2	1.6	<1.1	13.3	<2.0	1.0	<1.5
Geometric mean	0.38	<0.05	0.66	<0.14	8.4	<0.12	0.75	0.13

[a] < = No colonies on the plate.

TABLE 2. *Proportions of Veillonella and Neisseria species of the total cultivable flora present on teeth 1 and 24 hr after cleaning and in the saliva of 10 subjects*[a]

Subject	Per cent cultivable from tooth surface at				Per cent cultivable from saliva	
	1 hr		24 hr			
	Veillonella	*Neisseria*	*Veillonella*	*Neisseria*	*Veillonella*	*Neisseria*
A	<0.08	<0.08	0.2	0.03	2.4	0.2
B	7.3	1.6	0.4	7.8	15.0	7.0
C	<0.12	<0.12	0.1	6.5	7.5	7.5
D	5.7	3.3	0.08	0.4	13.5	17.0
E	<0.05	1.1	0.02	0.3	0.3	3.3
F	0.06	<0.06	0.2	0.02	22.0	1.7
G	1.0	<0.1	0.2	0.02	5.4	0.01
H	<0.2	<0.9	0.07	<0.05	2.7	<0.01
I	<0.05	<0.05	10.1	0.01	4.7	0.3
J	0.17	9.1	4.2	19.4	6.4	9.1
Arithmetic mean	<1.5	<1.5	1.6	<3.4	8.0	<4.6
Geometric mean	<0.27	<0.4	0.26	<0.24	5.0	<0.9

[a] < = No colonies on the plates.

Adherence of natural Veillonella and Neisseria species to cleaned tooth surfaces. To determine whether the low proportions of *Veillonella* and *Neisseria* species found on teeth reflected their relative inability to adhere to these surfaces, the proportions of these species adherent to teeth 1 and 24 hr after cleaning were studied. It was found that only low proportions of *Veillonella* and *Neisseria* species attached to teeth within 1 hr of cleaning (Table 2). This relatively short time interval tends to negate the possibility that other bacterial species were selectively growing on the teeth, thus lowering the proportions of *Neisseria* and *Veillonella* in relation to the total cultivable flora. The saliva of these subjects contained 10- to 20-fold higher proportions of *Veillonella* species (Table 2) than those found attached to the teeth. The differences in proportions of *Veillonella* in saliva compared to the tooth surfaces were statistically significant at the 1% level for 1-hr samples when analyzed by the Sign test. The relative proportions of *Neisseria* were two- to threefold greater in saliva than on tooth surfaces, but these differences were not statistically different. When these experiments were repeated with a 24-hr interval, the proportions of both *Neisseria* and *Veillonella* in plaque were also low. These data indicate that neither *Veillonella* nor *Neisseria* species is selectively adsorbed to cleaned teeth. Rather, these

TABLE 3. *Proportions of streptomycin-resistant Streptococcus sanguis, Streptococcus salivarius, Veillonella, and Neisseria species adhering to dental plaque of unknown age, the tongue dorsum, and the vestibular mucosa of five subjects*

Subject	Strain	Mixture introduced into mouth	Vestibular mucosa	Tongue dorsum	Plaque samples			
					1	2	3	4
A	*Veillonella* VC-R	24.8	11.4	48.7	1.1	0.3	0.2	2.2
	Neisseria ND-R	28.3	8.3	50.2	1.2	0.3	0.3	5.4
	S. salivarius 9GS2-R	11.6	1.3	0.6	1.3	0.1	0.7	0.6
	S. sanguis H7P-R	35.3	79.0	0.4	96.4	99.3	98.7	91.8
B	*Veillonella* VC-R	26.8	10.0	56.0	2.0	0.3	1.8	10.0
	Neisseria ND-R	14.2	11.0	11.0	5.0	1.4	5.0	11.0
	S. salivarius 9GS2-R	23.6	45.0	18.0	13.0	1.3	4.4	10.0
	S. sanguis Mi-R	35.4	34.0	15.0	80.0	97.0	89.0	68.0
C	*Veillonella* VC-R	14.0	12.0	51.0	0.1	0.1	0.6	0.4
	Neisseria ND-R	36.0	24.0	15.0	0.3	15.5	0.4	11.8
	S. salivarius 9GS2-R	9.0	45.0	29.0	1.6	2.2	1.7	11.5
	S. sanguis H7P-R	41.0	19.0	5.0	98.0	82.0	98.0	77.0
D	*Veillonella* VC-R	1.0	5.3	71.6	0.1	0.0	0.4	0.0
	Neisseria ND-R	8.7	14.7	16.3	0.8	0.0	1.9	0.0
	S. salivarius 9GS2-R	6.4	1.3	1.3	0.2	0.0	0.4	0.0
	S. sanguis Mi-R	84.1	78.7	10.9	98.9	100.0	97.4	100.0
	Veillonella VC-R	10.0	9.0	52.0	5.3	1.8	5.5	2.7
	Neisseria ND-R	17.6	1.4	9.4	5.1	1.8	4.7	9.5
	S. salivarius 9GS2-R	16.1	5.3	32.4	2.0	5.4	1.8	10.8
	S. sanguis Mi-R	56.2	84.2	6.3	87.6	91.0	88.0	76.9

organisms possess a weak adherence to teeth, which, in the case of *Veillonella* species, results in a 10- to 20-fold dilution in their proportions as compared to saliva.

Adherence of streptomycin-labeled Veillonella and Neisseria species to preformed dental plaque, tongue, and cheek. Because the surface properties of preformed dental plaque are likely to differ from those of teeth, the ability of *Veillonella* and *Neisseria* to adhere to the periphery of plaque was determined by using streptomycin-labeled strains. For comparative purposes, streptomycin-labeled strains of *S. sanguis* and *S. salivarius* were included in the mixtures. It was found that the proportions of labeled *Veillonella* and *Neisseria* recovered from the plaque samples were much lower than those present in the mixture introduced into the mouths of the five subjects studied (Table 3). In contrast, the proportions of labeled *S. sanguis* recovered from the plaque samples were several fold higher than those present in the bacterial mixture, confirming the marked adherence of this organism to plaque (van Houte et al., *unpublished data*). The adherence of *S. salivarius*, an organism present in plaque in only low proportions, was also very low, being comparable to that of the *Veillonella* and *Neisseria* strains studied.

The adherence of labeled *Veillonella* and *Neisseria* strains to oral epithelial surfaces was also studied. The adherence of *Veillonella* to the tongue surface was found to be high since much higher proportions of this organism were recovered from the tongue than were present in the original mixture (Table 3). The proportions of labeled *Neisseria* and *S. salivarius* recovered from the tongue were not clearly different from those of the original mixture. However, the proportions of *S. sanguis* in the tongue samples were appreciably lower than those present in the original mixture, indicating the weak adherence of this organism to the tongue surface. The proportions of all four organisms to the vestibular mucosa did not differ from those of the original mixture (Table 3).

DISCUSSION

Veillonella and *Neisseria* species have been reported to comprise part of the microbial populations found on teeth, in saliva, and on the tongue (6, 7, 10, 11, 13). In the present investigation, *Veillonella* species averaged 5 and 8% of the cultivable flora present in saliva and on the tongue surfaces, respectively, but these organisms averaged below 1% of the flora present in other sites in the mouth. The relatively high proportions of *Veillonella* in saliva and on the tongue supports the contention that the majority of salivary bacteria represent organisms which are washed off of the tongue surface (3, 9). When detected,

Neisseria cells were usually in low proportions (less than 1%) in all sites studied. The finding that *Veillonella* and *Neisseria* species were present in particularly low proportions in the microbial accumulations adhering to teeth either 1 or 24 hr after cleaning is in contrast to the reports of Ritz (12, 13). He reported that *Neisseria* species averaged between 9 and 18% of the aerobically cultivable flora present in early plaque samples and suggested that aerobic organisms such as *Neisseria* may uniquely affect the rate of plaque formation by facilitating later colonization by anaerobes. The present investigations used the selective medium and incubation techniques described by Ritz (12, 13) for enumeration of *Neisseria*. However, our data are expressed as a percentage of the flora cultivable on anaerobically incubated blood-agar, rather than on aerobic plates, since it is well known that anaerobically incubated plates give much higher colony counts for oral samples (4, 6, 7). The very low proportions of *Neisseria* present in 1- or 24-hr plaque samples do not support the contention that they are uniquely important in plaque development. In addition, the anaerobic *Veillonella* were found to be present in approximately the same proportion in the samples studied, suggesting that aerobic organisms are not necessarily favored in early plaque nor are they prerequisites for the presence of anaerobes.

It has recently been shown that bacteria differ widely in their abilities to adhere to enamel and oral epithelial surfaces both in vitro and in vivo. It has been found that *S. salivarius* adheres to oral epithelial surfaces much better than to tooth surfaces, and this difference in adherence correlates with the natural occurrence of this organism in the mouth (5, 8, 15; van Houte et al., *unpublished data*). Similarly, the adherence of *S. sanguis* and *Streptococcus mutans* for teeth and oral epithelial surfaces also correlates with their natural distribution in the oral cavity. Therefore, the ability of bacteria to attach to surfaces seems to be a major ecological determinant influencing colonization in environments which contain surfaces exposed to bathing fluids. Organisms which cannot attach to a surface are simply washed away. Since bacteria differ widely in their ability to attach to various surfaces, these differences would seem likely to influence the extent to which they can colonize. The present investigation has shown that *Veillonella* species attach well to the tongue surface, their adherence being greater than that of *S. sanguis*, *S. salivarius*, and *Neisseria* species. However, *Veillonella* were not found to adhere well to teeth and cheeks. Their adherence thus reflects their proportions found indigenously on the tongue in relation to the other organisms and other oral surfaces studied. *Neisseria* species were not observed to have an unusual affinity for any of the oral surfaces studied, and these organisms were found to comprise less than 1% of the total cultivable flora present in these sites. Thus, the ability of *Veillonella* and *Neisseria* species to attach to oral surfaces correlates well with their natural occurrence. It would appear that bacterial adherence to surfaces is of major ecological importance for at least some gram-negative bacteria in the mouth in addition to the gram-positive streptococci studied previously.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant DE-02847 from the National Institute of Dental Research and by a grant from the Colgate-Palmolive Co. One of the authors (W. L.) was supported by a special research fellowship from the National Institutes of Health.

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