Bull. Tokyo Med. Dent. Univ., 28: 117–123, 1981

ISOLATION AND ENUMERATION OF MYCOPLASMAS IN DENTAL PLAQUES

BY

Akihisa UCHIDA*1

ABSTRACT

There has been only a fragmentary knowledge concerning mycoplasmas in the dental plaques and, in particular, no quantitative studies have been made on them. For this reason, isolation and enumeration of the mycoplasmas in the dental plaques have been attempted in this present study.

The incidence of mycoplasmas was significantly higher in the dental plaques accumulated on the molars (76.1%, 35/46 specimens) than on the incisors (37.2%, 16/43); on the healthy enamel surface (hereafter called normal plaques; 61.0%, 61/100) than on the superficially decayed enamel surface (hereafter called caries plaques; 14.8%, 4/27) and on the healthy cervical enamel surface but in contact with the inflamed gingival marginal area (hereafter called gingivitis plaques; 30.3%, 10/33).

The number of organisms (cfu) in the dental plaques (mg) was greater in the normal plaques (range, $0-1.57\times10^6$: mean, 6.31×10^4) than in the caries ($0-4.62\times10^2$: 2.28×10^2) and in the gingivitis ($0-4.14\times10^4$: 6.55×10^3). There was a significant difference only between the normal and caries plaques. In addition, the number of organisms in the 1-day and 3-day-old dental plaques suggested that the mycoplasmas were not one of the microorganisms which appeared at the early stages of dental plaque formation.

M. salivarium (311 strains) and M. orale (13) were isolated from 67 samples of dental plaques, but U. urealyticum was not. Of the 67 samples, 60 (89.5%), 1 (1.5%) and 6 (9.0%) samples were positive for M. salivarium, M. orale and both of these two species, respectively.

INTRODUCTION

Since the first successful isolation of mycoplasmas from the human saliva was reported in 1951 by Morton et al. [1]; further studies have shown a wide distribution of mycoplasmas in the human oral cavity (Morton et al. [2]; Burnett and Gilmore [3]; Barile and Sheingorn [4]; Itoh [5]; Takeuchi [6]; Shklair et al. [7]; Razin et al. [8]; Serene and Anderson [9]; Watanabe [10–12]; Kumagai et al. [13]; Ōhashi [14]).

The frequency of the isolation of mycoplasmas from the oral cavity is very high and most of the mycoplasma strains isolated are identified as M. salivarium and M. orale. These findings are almost common to those described by the investigators cited above, even though the methods of sampling, growth media and cultural conditions applied are different. However, it seems that there has been only a fragmentary knowledge concerning the organisms in the dental plaques, because attention by most of the investigators, so far, has been directed to mainly the organisms in the saliva, washings of the mouth, deposits in the gingival pockets and so on. Therefore, little data are available concerning mycoplasmas in the well-defined dental plaques sampled under the condition that contamination by saliva, sulcular fluid and deposits in the gingival pockets was excluded as much as possible, and, especially, the number of organisms in such dental plaques.

*1 内田明比佐: Department of Oral Microbiology (Chief: Prof. T. HORIKAWA), Faculty of Dentistry, Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku).

Received for publication, September 1, 1981.

In the present study, enumeration and isolation of mycoplasmas in the dental plaques were attemped.

Materials and Methods

Subjects: Subjects were 130 male and female patients ranging from 7 to 77 years of age who visited the clinic of the 1st Department of Oral Surgery, Faculty of Dental Medicine, Tsurumi University.

Sampling techniques: First, the subjects were requested to gargle with a glass of water. The teeth, from which the dental plaques were sampled, were washed very well by spraying with a large amount of water and dried with a sterile gauze sponge after isolating the teeth with a sterile cotton and washed once again with sterile saline. The dental plaques were taken by scraping with a sterile steel applicator. Additionally, in order to see the shift of the number of viable mycoplasma organisms in the developing dental plaques, the dental plaques at 2 different stages of development were obtained from 12 subjects as follows. After the buccal surface of the upper 1st molars (left and right) of each subject was given a dental prophylaxis to remove all previously existing dental plaques, the subjects were asked to refrain from brushing the previously cleaned surface of the tooth during the experimental period. From the same subject, the dental plaques were sampled from the surface of the right tooth after 1 day and the left tooth after 3 days. Care was taken to avoid sampling materials from the gingival crevices or the proximal spaces.

Media: Media were those used in the previous study (Uchida et al. [15]). However, for the growth of ureaplasmas, the liquid medium was supplemented with urea (0.1%, w/v) instead of arginine-HCl (1.0%, w/v), and the reaction of the agar medium, after being supplemented with urea (0.01%, w/v), was adjusted to pH 6.0. The concentration of thallium acetate was changed from 1:2000 to 1:4000.

Isolation and enumeration of mycoplasmas: Isolation and enumeration of the mycoplasmas in the dental plaques were performed according to the techniques described in the previous report (Uchida et al. [15]). And the following method was also applied to isolate the mycoplasmas from the dental plaques. The dental plaques were suspended in the ureaplasma medium (5 ml) contained in the screw-capped vials and shaken vigorously for 2 minutes using the Thermo-Mixer Model TM-100 (Thermonics Co., Ltd.). Then the suspensions were incubated statically at 37°C for 2 days. The procedure after this is just the same as described in the previous report (Uchida et al. [15]).

Identification of mycoplasmas: Isolates were identified by growth inhibition (Clyde [16]) or metabolic inhibition test (Purcell et al. [17]) using hyperimmune rabbit antisera against M. salivarium ATCC 23064 and M. orale ATCC 15539 prepared in our laboratory and stored at—70°C.

Results

Incidence of mycoplasmas in the dental plaques: First of all, the frequency of isolation of mycoplasmas from the dental plaques sonicated and that from those shaken vigorously were compared to see how sonication and shaking affected the frequency of isolation of the mycoplasmas from the dental plaques. However, both of these techniques were found not to make any significant differences in the frequency of isolation of mycoplasmas. Mycoplasmas were isolated from 37% (16/43 specimens) of the dental plaques accumulated on the incisors and from 76% (35/46 specimens) of those on the molars. In addition, the incidence of mycoplasmas

Table 1. Incidence of Mycoplasmas in Dental Plaques Accumulated on Buccal Enamel Surface of Incisors and Molars

Plaque	Incisor	Molar	P Value[1]	Unidentified[2]	Total	P Value[1]
Normal	* 8/18(44.4%)	*59/82(72.0%)	NT		*67/100(67.0%)	
	**16/43(37.2%)	**35/46(76.1%)	<0.01[3]	**10/11(90.9%)	**61/100(61.0%)	—
Gingivitis	** 5/23(21.7%)	** 5/10(50.0%)	NT		**10/ 33(30.3%)	<0.01[4]
Caries	** 2/ 9(22.2%)	** 2/18(11.1%)	NT		** 4/ 27(14.8%)	<0.001[5]

1. Analyzed by chi-square test. 2. Dental plaques the origin of which can not be identified. 3. For incisors versus molars. 4. For normal versus gingivitis. 5. For normal versus caries.
* Plaques shaken. ** Plaques sonicated. NT: Not tested.

Table 2. Number (cfu/mg) of Mycoplasmas in Dental Plaques

Plaque	No. of specimens	Range	Mean	P Value*
Normal	100	$0-1.57\times10^6$	6.31×10^4	—
Gingivitis	33	$0-4.14\times10^4$	6.55×10^3	NS
Caries	27	$0-4.62\times10^2$	2.28×10^2	<0.05**

* Analyzed by Wilcoxon's rank sum test. ** For normal versus caries.
NS: Not significant.

in the dental plaques accumulated on the healthy enamel surface (hereafter called normal plaques), superficially decayed enamel surface (hereafter called caries plaques) and on the healthy, cervical enamel surface and in contact with the inflamed gingival marginal area (hereafter called gingivitis plaques) were 61% (61/100 specimens), 30% (10/33 specimens) and 15% (4/27 specimens), respectively. By statistical analysis of these results, the incidence of mycoplasmas was significantly higher in the dental plaques accumulated on the molars than on the incisors and in the normal plaques than in the caries and gingivitis plaques (Table 1).

The number of mycoplasma organisms in the dental plaques: One hundred, 33 and 27 specimens of normal, gingivitis and caries plaques, respectively, were examined for viable counts of mycoplasmas. Normal, gingivitis and caries plaques were demonstrated to contain mycoplasmas with mean counts of 6.31×10^4 cfu/mg, 6.55×10^3 cfu/mg and 2.28×10^2 cfu/mg,

Table 3. Number of Mycoplasmas (cfu) in Developing Dental Plaques (mg, Wet Weight) on the Buccal Surface of Upper First Molars

Subject No.	Days 1	3
1	1.50×10^3	3.81×10^2
2	0.00	5.45×10^2
3	0.00	0.00
4	3.33×10^2	0.00
5	0.00	3.80×10^3
6	6.82×10^3	4.00×10^4
7	2.84×10^2	1.54×10^2
8	0.00	0.00
9	0.00	0.00
10	0.00	0.00
11	8.57×10^2	9.92×10^3
12	0.00	1.22×10^4

Table 4. Mycoplasma Species Isolated from Dental Plaques

Number of strains identified with		
M. salivarium	M. orale	U. urealyticum
311 (96.0%)	13 (4.0%)	0 (0.0%)

Table 5. Mycoplasma Species Isolated from Dental Plaques

Incidence of the following mycoplasmas in dental plaques*		
M. salivarium	M. orale	M. salivarium+M. orale
*60/67(89.5%)	*1/67(1.5%)	*6/67(9.0%)

* No. of positive samples/No. of samples tested

respectively, ranging from 0 to 1.57×10^6 cfu/mg, 0 to 4.14×10^4 cfu/mg and 0 to 4.62×10^2 cfu/mg. There was a significant difference between the viable counts in the normal plaques and those in the caries plaques but no significant difference between those in the normal and gingivitis plaques and between those in the gingivitis and caries plaques (Table 2).

The number of mycoplasma organisms in the dental plaques at 2 different stages of development: The number of mycoplasma organisms in the 1-day-old and 3-day-old dental plaques was measured. The viable counts increased obviously in 4 subjects (Subject No. 2, 5, 11 and 12) and very slightly in one subject (Subject No. 6) but did not attain the mean (6.31×10^4 cfu/mg). In 4 subjects, mycoplasmas could not be detected from both of the 1-day-old and 3-day-old plaques. In the remaining 3 subjects (Subject No. 1, 4 and 7), the viable counts decreased in a 2-day time, although the decrease in Subject No. 1 and 7 might not be significant (Table 3).

Identification of mycoplasma strains isolated from the dental plaques: A total of 344 mycoplasma strains were isolated from 67 samples of dental plaques. Of all isolates, 311 and 13 strains were identified as M. salivarium and M. orale, respectively. Ureaplasmas were not isolated at all (Table 4). Of the 67 samples of the dental plaques, 60 (85.5%), 1 (1.5%) and 6(9.0%) samples were shown to be positive for M. salivarium, M. orale and both of these 2 species, respectively (Table 5).

Discussion

Mycoplasmas are almost always contained in the saliva, sulcular fluid and deposits in the gingival pockets (Watanabe [10]; Kumagai et al. [13]; Engel et al. [18]; Forest [19]). Therefore, in the present study, the dental plaques were sampled very carefully not to be contaminated by them, because contamination by them was considered to raise the frequency of isolation of mycoplasmas from the dental plaques and to cause the indigenous mycoplasmal flora in the dental plaques to be misinterpreted. Consequently, contamination of the normal and caries plaques by the saliva and deposits in the gingival pockets were presumed to be excluded to a considerable extent, as these two plaques were sampled from the sites which could be separated very easily from the circumstances surrounding them. But the gingivitis plaques might be contaminated inevitably a little bit with sulcular fluid and deposits in the gingival pockets.

Mycoplasmas were isolated from 128 of 200 samples (64%) of the dental plaques. The frequency (64%) of isolation was far higher than that (14.9%, 17/47) described by Kumagai et al. (13). The incidence of mycoplasmas was significantly higher in the dental plaques on the molars than those on the incisors. This result may be reasonable, because the anaerobic condition favorable for the growth of mycoplasmas is supposed to be fullfilled much better in the posterior teeth anatomically than in the anterior teeth. The number of

organisms was greater in the dental plaques on the molars than that on the incisors. However, there was no significant difference between them.

Dental plaques are closely associated with dental caries and periodontal diseases from the etiological point of view. And it is demonstrated that the preferential site of M. salivarium in the oral cavity is the gingival sulci or gingival pockets (Kumagai et al. [13]). Besides, there are some findings that suggest the association of the organisms with periodontal diseases etiologically. (Watanabe et al. [12]; Kumagai et al. [13]; Forest [19]; Parkinson and Carter [20]; Parkinson [21]). For this reason, the author was interested in knowing the incidence and the number of mycoplasmas in the pathological dental plaques, because knowledge concerning mycoplasmas in such dental plaques is extremely limited. The incidence and the number of organisms were surprisingly and unexpectedly less in the dental plaques accumulated on the healthy cervical enamel surface and in contact with the inflamed gingival marginal area and on the superficially decayed enamel surface than in those on the healthy enamel surface. Physiological conditions provided by the microbial flora constituting the dental plaques and interactions taking place between the members of the flora may be more favorable for the mycoplasmas in the normal dental plaques than in the pathological ones. One of the very important factors influencing the growth of mycoplasmas is pH. The optimal pH for them is rather alkaline at about 7.8. Pathological dental plaques dominated by bacteria hydrolyzing sugars actively and producing acids may not be agreeable as the habitat of the organisms.

Both M. salivarium and M. orale were detectable from the dental plaques, although M. orale accounted for only 4% of all isolates. This finding is not in agreement with the other investigator's (Kumagai et al. [13]) description that isolates from the dental plaques were identified as M. salivarium exclusively. However, it is worthy of note that 7 (10.5%) of 67 dental plaques contained M. orale, although 6 samples of these contained M. salivarium as well. In the present study, normally, 5 strains were isolated from each of the samples, which made it possible to isolate the mycoplasmas of the plural species. It is interesting that M. salivarium is so predominant in the dental plaques in spite of the fact that saliva contains M. orale as well as M. salivarium and the incidence of M. orale in the saliva ranges from 20 to 60% depending on the clinical conditions in the oral cavity according to our observations (unpublished data, to be published). It is also interesting to know the reason why M. salivarium is so predominant in the dental plaques. Previously, the author (Uchida et al. [15]) found that M. salivarium was markedly superior to M. orale in regard of the attachment of the cells to the glass and plastic surface. This property may elucidate part of the reason.

Ureaplasmas were originally isolated from the urethra by Shepard [22] and afterwards isolated mainly from the urogenital tract by many investigators (Taylor-Robinson and McCormack [23]). Recently, the author and his colleagues (unpublished data, to be published) isolated ureaplasmas from the saliva sampled from patients afflicted with periodontal diseases, pericoronitis and so on, although the incidence was very low (3.5%). The author was interested in knowing whether U. urealyticum was a member of the oral microbial flora and attempted to isolate the organisms from the dental plaques, but failed. As ureaplasmas are rather fastidious, this failure is not considered to imply that the organisms do not inhabit the den-

tal plaques.

There have been few cultural studies on the developing dental plaques. Ritz [24] observed that the proportion of Streptococcus, Nocardia and Neisseria decreased, while that of Veillonela, Fusobacterium and Actinomyces increased during the dental plaque development. The shifts in the population of Streptococcus and Actinomyces have been observed by Loesche and Syed [25], and Syed et al. [26], while Theilade and Theilade [27] found gram-positive cocci, particularly Streptococcus, to be dominant in the 1-day-old and 3-day-old plaques. The author measured the number of mycoplasma organisms in the 1-day-old and 3-day-old plaques. As the number of subjects was limited, it was difficult to say something definite. But the results obtained seemed to suggest that mycoplasmas were not one of the micro-organisms which might appear in the early stages of the development of dental plaques.

Acknowledgements

The author is sincerely grateful to Prof. T. Horikawa of the Department of Oral Microbiology, Faculty of Dentistry, Tokyo Medical and Dental University, for his valuable advices and guidance during the course of this study. The author is indebted also to Prof. H. Fujita of the Department of Bacteriology for offering generously the opportunity to carry out this study in his laboratory and to Prof. K. Seto and Asst. Prof. M. Matsuura of the 1st Department of Oral Surgery, Faculty of Dental Medicine, Tsurumi University, for collecting the samples from the patients. The author thanks also the staff of the 1st Department of Oral Surgery, Faculty of Dental Medicine, Tsurumi University, for participating in some of the experiments as subjects and Prof. T. Watanabe of the Department of Oral Bacteriology, Nagasaki University School of Dentistry, for his advices and discussion on this paper.

References

[1] Morton, H. E., Smith, P. F., Williams, N. B., and Eickenberg, C. F.: Isolation of PPLO from human saliva: A newly detected member of the oral flora. J. Dent. Res., 30: 415–422, 1951.

[2] Morton, H. E., Smith, P. F., and Keller, R.: Prevalence of PPLO and the evaluation of media and methods for their isolation from clinical material. Am. J. Pub. Health, 42: 913–924, 1952.

[3] Burnett, G. W., and Gilmore, E.: Incidence and distribution of oral PPLO and L-organisms. J. Dent. Res., 38: 632, 1959.

[4] Barile, M. F., and Sheingorn, A.: The isolation and cultivation of a filterable form resembling PPLO from the human dental pulp. O. S. O. M. O. P., 13: 756–760, 1960.

[5] Itoh, S.: Studies on the distribution of pleuropneumonia-like organisms (PPLO) among human and animals (in Japanese). Japan. J. of Bacteriol., 15: 1193–1199, 1960.

[6] Takeuchi, T.: Isolation of PPLO from the human oral cavity (in Japanese, with English summary). Shika Gakuho (J. Tokyo Dent. Coll. Soc.), 62: 305–315, 1962.

[7] Shklair, I. L., Mazzarella, M. A., Gutenkunst, R. R., and Kiggins, E. M.: Isolation and incidence of PPLO from the human oral cavity. J. Bacteriol., 83: 785–788, 1962.

[8] Razin, S., Michmann, J., and Shimshoni, Z.: The occurrence of mycoplasma (pleuropneumonia-like organisms, PPLO) in the oral cavity of dentulous and edentulous subjects. J. Dent. Res., 43: 402–405, 1964.

[9] Serene, T. P., and Anderson, D. L.: Isolation of mycoplasma from human root canals. J. Dent. Res., 46: 395–399, 1967.

[10] Watanabe, T.: Biological and serological studies on oral mycoplasma. Bull. Tokyo Med. Dent. Univ., 14: 447–460, 1967.

[11] Watanabe, T., Ōhashi, Y., Takamori, K., and Horikawa, T.: Kōkū kara bunri shita mycoplasma ni tsuite (Studies on mycoplasmas isolated from oral cavities) (in Japanese). Japan. J. of Bacteriol., 24: 578–579, 1969. (Proceedings).

[12] Watanabe, T., Mishima, K., Fujita, O., Horikawa, T., Noguchi, T., Ishizu, T., and Kinoshita, S.: Possible role of mycoplasmas in periodontal disease. Bull. Tokyo Med. Dent. Univ., 19: 93–109, 1972.

[13] Kumagai, K., Iwabuchi, T., Hinuma, Y., Yuri, K., and Ishida, N.: Incidence, species, and

significance of mycoplasma species in the mouth. J. Inf. Dis., 123: 16–21, 1971.

[14] Ōhashi, Y.: Studies on oral mycoplasma in relation to periodontal disease (in Japanese, with English summary). J. Japan. Stomatol. Soc., 38: 363–374, 1971.

[15] Uchida, A., Horikawa, T., Matsuura, M., and Watanabe, T.: A preliminary study on isolation and enumeration of mycoplasmas in dental plaques: The effect of sonication on viability of oral mycoplasmas. Bull. Tokyo Med. Dent. Univ., 28: 111–116, 1981.

[16] Clyde, W. A.: Mycoplasma species identification based upon growth inhibition by specific antisera. J. Immunol., 92: 958–965, 1964.

[17] Purcell, R., Taylor-Robinson, D., Wong, D., and Chanock, R. M.: A color test for the measurement of antibody to the non-acid-forming human mycoplasma species. Am. J. Epidemiol., 84: 51–66, 1966.

[18] Engel, L., and Kenny, G. E.: Mycoplasma salivarium in human gingival sulci. Periodont. Res., 5: 163–171, 1970.

[19] Forest, N.: Caractérisation de mycoplasma salivarium dans les parodontopathies. J. Biol. Buccale, 7: 321–330, 1979.

[20] Parkinson, C. F., and Carter, P. B.: Phagocytosis of mycoplasma salivarium by human polymorphonuclear leukocytes and monocytes. Infect. Immun., 11: 405–414, 1975.

[21] Parkinson, C. F.: Histamine release from human leukocytes when stimulated by Mycoplasma salivarium. Infect. Immun., 11: 595–597, 1975.

[22] Shepard, M. C.: The recovery of pleuropneumonia-like organisms from Negro men with and without nongonococcal urethritis. Am. J. Syph. Gonor. Vener. Dis., 38: 113–124, 1954.

[23] Taylor-Robinson, D., and McCormack, W.: The genital mycoplasmas. N. Engl. J. Med., 302: 1003–1010, 1063–1067, 1980.

[24] Ritz, H. L.: Microbial population shifts in developing human dental plaque. Arch. Oral Biol., 12: 1561–1568, 1967.

[25] Loesche, W. J., and Gibbons, R. J.: A practical scheme for identification of the most numerous oral gram-negative anaerobic rods. Arch. Oral Biol., 10: 723–725, 1965.

[26] Syed, S. A., Loesche, W. J., and Löe, H.: Bacteriology of dental plaque in experimental gingivitis. 2. Relationship between time, plaque score and flora. A. A. D. R. Abstract #109, 1975.

[27] Theilade, E., and Theilade, J.: Bacteriological and ultrastructural studies of developing dental plaque. In "Dental Plaque", McHugh, W. D., ed. E. S. Livingstone, Edinburgh, 1970, pp. 27–40.