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Sonographic diagnosis for Mikulicz disease

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Objective. The aim was to investigate the diagnostic imaging characteristics of Mikulicz disease (MD), especially sonographic ones, and to clarify the differences between them and those in Sjögren syndrome (SS), based on new criteria of MD.
Study design. The sonographic and sialographic images, as well as clinical, histopathologic, and serologic findings of 9 patients satisfying the new criteria of MD were analyzed and compared with those in SS.
Results. All swollen submandibular glands showed bilateral nodal hypoechoic areas with high vascularization on sonograms and a parenchymal defect on sialograms, whereas parotid glands showed normal or slight change on both images. Nodal areas in submandibular gland sonograms were unclear on computerized tomography and on magnetic resonance imaging, but showed accumulation on gallium scintigraphy.
Conclusion. Mikulicz disease showed a high rate of bilateral nodal change in submandibular glands, which was completely different from SS. For detection and follow-up of these changes, sonography may be the best imaging modality. **(Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2009;108:105-113)**

Mikulicz disease (MD) was first reported by Johann von Milulicz-Radecki in 1888 as a disease of unknown etiology with symmetric swelling of bilateral lacrimal and salivary glands.[1] In 1927, Schaffer and Jacobsen[2] classified the disease into 2 types based on whether patients had certain underlying diseases, such as leukemia, malignant lymphoma, sarcoidosis, tuberculosis and syphilis, or whether they did not have any such diseases. In the former case, the illness was named "Mukulicz syndrome," and in the latter case "Mikulicz disease." In 1953, Morgan and Castleman[3] analyzed histopathologic findings of 18 cases of MD and concluded that MD was a less highly developed subtype of Sjögren syndrome (SS). After that, however, there were very few reports about MD for about a half-century.

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Regarding imaging examination, sialograms of MD and those of SS have been reported as having similar punctate or globular sialectasis in periphery.[4-9] Only 1 report demonstrated the sialographic differences between MD and SS.[10] It reported none of the punctate or globular sialectasis typically seen in SS, but demonstrated a defect of the parenchymal image in MD. Unfortunately, because the criteria of MD were still confused with those of SS, that report did not attract much attention.

More recently, however, Tsubota et al.[11] reported that the frequency of gland cell apoptosis in MD was significantly lower compared with that in SS. Furthermore, Yamamoto et al.[12,13] announced the clinical and serologic differences between SS and MD. These differences include persistent gland swelling in MD, whereas that in SS was periodic. Moreover, salivary function was either normal or improved with the administration of glucocorticoid in MD, whereas salivary function decreased and was not affected by treatment in SS. A further difference showed marked elevation of the level of immunoglobulin (Ig) G4 in MD serum although it tested negative for antiSS-A and/or antiSS-B antibodies, whereas SS showed normal IgG4 level but a high positive rate for antiSS-A and/or antiSS-B antibodies. Prominent infiltration of IgG4-positive plasmacytes was observed with immunostaining in MD, whereas there were no IgG4-positive plasmacytes seen in SS. In addition, no punctate or globular sialectasis was observed on sialograms in MD, whereas they were generally observed in SS. Therefore, Yamamoto et al.[14] considered MD to be an

Table I. Criteria for Mikulicz disease

Essential requirements:
1. Bilateral persistent swelling during more than three months in at least one of the lacrimal, parotid, and submandibular glands.
2. Lymphocyte infiltration pattern shows that of benign lymphoepithelial lesion on histopathologic studies.

IgG4 requirements:
1. Increased level of IgG4 in the serum.
2. Infiltration of IgG4-positive plasmacytes on histopathologic studies.

Other conditions:
1. Mild decrease in saliva secretion.
2. Low positive rates of antiSS-A and/or antiSS-B antibody in the serum.
3. No punctuate or globular pattern on sialograms.
4. High steroid sensitivity.

Ig, Immunoglobulin.

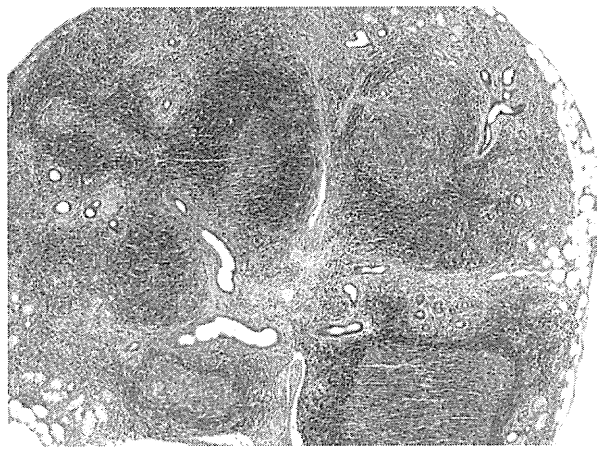


Fig. 1. Histopathological section (HE stain) of a labial gland of Case 7 (under low magnification). The section shows extensive replacement of gland parenchyma by lymphoid follicles. Periductal lymphocytic infiltration is relatively modest compared with Sjögren syndrome.

entity independent of SS. They also gave a brief description of diagnostic imaging of MD; however, details of the imaging characteristics of this disease have not yet been reported.

The purpose of the present study was to investigate the diagnostic imaging characteristics of MD, especially the sonographic ones, and to clarify the differences between those characteristics and those of SS, based on the new criteria of MD.

MATERIALS AND METHODS

Criteria of MD

Although unanimous criteria for MD have not been defined so far, they are being discussed in the working group for MD of the Japanese Medical Society for SS. Our institution's criteria are listed in Table I. There are 2 conditions that are essential for an ailment to be considered as MD. The first is persistent bilateral swelling in ≥ 1 of the lacrimal, parotid, and submandibular glands for >3 months. SS shows repeated swelling or swelling that disappears in due course. The second condition is that the lymphocyte infiltration pattern should show that of benign lymphoepithelial lesion on histopathologic studies. Benign lymphoepithelial lesion is characterized by lymphocytic infiltration of the salivary glands, similar to the germinal center, and is further characterized by destruction or replacement of the acini with the persistence of islands of epithelial cells (Fig. 1). In contrast, SS shows mainly periductal infiltration of lymphocytes. Regarding IgG4, MD generally shows: 1) increased level of IgG4 in the serum; and 2) Infiltration of IgG4-positive plasmacytes with immunostaining on the histopathologic studies. Other characteristics of MD include the following conditions:

1. Mild decrease in saliva secretion.
2. Low positive rates of antiSS-A and/or antiSS-B antibody in the serum.
3. No punctuate or globular pattern on sialograms.
4. High steroid sensitivity.

In the present study, when a case satisfied the 2 essential conditions, it was considered to be MD, even if it did not satisfy other supplemental conditions.

Patients

We retrospectively analyzed patients who visited our hospital from January 1999 to July 2007 with suspected MD or complaints of salivary gland swelling. In this study the patients who satisfied the 2 essential requirements mentioned in the preceding section were analyzed. The subjects comprised 9 patients: 7 women and 2 men, with a mean age of 56.9 years (range 31 to 68 years).

Clinical findings

We checked the location and duration of persistent bilateral swelling (essential requirement 1), general problems, complications, and the presence of dryness of mouth and eyes. All the patients underwent gum tests and/or Saxon tests for objective assessment of mouth dryness. When the patients had dryness of eyes, the Schirmer test was added for objective assessment. Normal values for the gum test and the Saxon test are >10 mL/10 min, and >2 g/2 min, respectively. Normal values for the Schirmer test are >10 mm/5 min, and a value of ≤ 5 mm is diagnosed as dry eyes. MD shows a mild decrease in saliva secretion (other condition 1),

whereas SS generally shows below-normal levels in objective assessment value of dryness.

Histopathologic examinations

Materials by labial gland biopsy were stained with hematoxylin and eosin, and were evaluated based on the scores reported by Chisholm and Mason.[15] They defined a focus as an aggregate of >50 lymphocytes and histiocytes, and 5-level grading was performed according to the number of foci seen on labial gland biopsy material per 4 mm^2 as follows: grade 0: lymphocytes were absent; grade 1: slight infiltration; grade 2: moderate infiltration or <1 focus; grade 3: 1 focus; and grade 4: >1 focus. Besides this grading, the pattern of lymphocytes infiltration, whether intralobular (pattern of benign lymphoepithelial lesion) or periductal (pattern of SS), was evaluated (essential requirement 2).

Serologic examinations

Serologic analysis was performed on rheumatoid factor (RF), antinuclear antibody (ANA), IgG, IgG4, IgA, IgM, and antiSS-A and antiSS-B antibodies. Normal values were as follows: RF: <20 immunizing units (IU)/mL; IgG: 872-1,815 mg/dL; IgG4: 4.8-135 mg/dL; IgA: 95-405 mg/dL; and IgM: 59-269 mg/dL. Levels of ANA were divided into 4 grades: negative: <40; 1+: 40-160; 2+: 160-640; and 3+: >640. AntiSS-A and antiSS-B antibodies were evaluated as positive when minute amounts of them were detected. Although MD shows high values in IgG and IgG4 (IgG4 requirement 1), it shows low positive rates in antiSS-A and/or antiSS-B antibodies (other condition 2).

Sonographic examinations

Sonographic examinations were performed and evaluated by 6 certified oral and maxillofacial radiologists, with more than 10 years of sonographic experience each, using an Acuson Sequoia 512 (Mochida Siemens Medical Systems, Tokyo, Japan). B-Mode multifoci images were taken with a center frequency of 8 MHz. Doppler-mode images were taken with a center frequency of 7 MHz and a flow range of 0.023 m/s. The bilateral parotid glands of each patient were scanned in 2 planes: parallel to the Frankfort-horizontal plane at the infra-auricular level and parallel to the retromandibular plane. The bilateral submandibular glands of each patient were scanned in 2 planes: one parallel and the other perpendicular to the submandibular plane. These are the standardized planes for salivary glands.[16]

We analyzed sonograms to determine whether there were findings compatible with SS on B-mode or other characteristic features. The findings compatible with SS on B-mode sonograms are stated in detail in our previous study,[17] which analyzes 79 suspected SS cases (including 43 actual cases). Compatible findings include multiple hypoechoic areas in the parotid gland and/or submandibular gland. These areas are often surrounded with hyperechoic lines and/or spots in typical SS cases. Obscuration of the gland configuration in the submandibular gland is also observed.

Sialographic examinations

Sialography was performed with a water-soluble contrast medium, amidotrizoate 76% (Urografin 76%; Schering-Japan, Osaka, Japan). Sialographic images were evaluated by a single observer (M.S.), based on the classifications by Rubin and Holt[18]: normal: no abnormal dilatation of the peripheral ducts; punctate: diffuse punctate dilatation of the peripheral ducts <1 mm; globular: the globules of contrast material increase to 1-2 mm; cavitary: the globules become irregular in size and distribution, cystic dilatation; and destructive: destruction of the gland parenchyma. MD shows generally normal sialograms (other condition 3), whereas SS shows an abnormal sialographic pattern in the classifications by Rubin and Holt at high rates.

Other imaging modalities

All of the patients underwent at least one of computerized tomography (CT), magnetic resonance imaging (MRI), and gallium scintigraphy under suspicion of, or to rule out, malignant lymphoma. When these images included salivary glands, we compared the findings with those of sonography.

Follow-up examinations

A follow-up CT or MRI was performed mainly for complications. When the patients showed persistent swelling of salivary glands, sonography was performed to assess the salivary gland condition.

RESULTS

Clinical findings

Table II shows the clinical findings of 9 cases. All cases showed bilateral swelling over the course of >3 months in ≥1 of the lacrimal, parotid, or submandibular glands (essential requirement 1). One case showed the swelling of lacrimal glands only, and 2 cases showed solely submandibular gland swelling. Five of the 9 cases showed swelling in lacrimal glands. Only 2 cases showed swelling in parotid glands, whereas in almost all (8 cases), submandibular glands showed swelling.

Six cases suffered from dryness of mouth; however, only one-half of them showed an objective decrease of saliva flow in both gum and Saxon tests. The average values of the gum and Saxon tests of 9 cases were 9.6 mL/10 min and 2.97 g/2 min, respectively. This result

Table II. Clinical findings

Case no.	Location of swelling					Duration (mo)	General problems	Complications	Dryness		Gum test (mL/10 min)	Saxon test (g/2 min)	Schirmer test (right/left, mm/5 min)
	LG	PG	SMG	SLG	PLG				Mouth	Eyes			
1	+					3	Fatigue	Asthma	+	+	4.0	3.76	8/4
2	+		+	+		18	Weight loss	AIP, DM	+		8.0	3.05	
3	+		+	+		3	Joint pain	—	+		13.0	2.73	
4	+		+	+	+	36	Weight loss	AIP, DM	+		7.8	1.03	
5		+	+	+		24	—	Prostate hypertrophy			16.0	5.80	
6			+			5	—	Hydronephrosis			12.0	4.26	
7	+		+			6	Anemia	AIP, PSC			9.8	4.33	
8			+			3	Weight loss	AIP, PSC	+	+	6.3	1.20	3/1
9		+	+			12	—	AIP, PSC	+		ND	0.60	

LG, Lacrimal glands; *PG*, parotid glands; *SMG*, submandibular glands; *SLG*, sublingual glands; *PLG*, palatal glands; *AIP*, autoimmune pancreatitis; *DM*, diabetes; *PSC*, primary sclerosing cholangitis; *ND*, not done.

Table III. Histopathologic and serologic findings

Case no.	Histopathologic findings		Serologic findings							
	Grade	Pattern	RF	ANA	IgG	IgG4	IgA	IgM	AntiSS-A	AntiSS-B
1	4	BLEL	−	+	high	ND	WNL	WNL	−	−
2	4	BLEL	−	+	high	ND	WNL	WNL	−	−
3	2	BLEL	−	−	high	ND	WNL	WNL	−	−
4	4	BLEL	−	2+	high	ND	WNL	WNL	−	−
5	4	BLEL	ND	−	ND	ND	ND	ND	−	−
6	4	BLEL	−	−	high	high	WNL	WNL	−	−
7	4	BLEL	−	−	high	high	WNL	WNL	−	−
8	4	BLEL	−	−	high	high	WNL	WNL	−	−
9	3	BLEL	+	2+	high	high	WNL	WNL	−	−

BLEL, Benign lymphoepithelial lesion; *RF*, rheumatoid factor; *ANA*, antinuclear antibody; *Ig*, immunoglobulin; *ND*, not done; *WNL*, within normal limits.

shows that the decrease of saliva flow was relatively mild in MD (other condition 1).

Frequent complications of MD were autoimmune pancreatitis (5 cases), primary sclerosing cholangitis (3 cases), and diabetes (2 cases).

Histopathologic and serologic findings

Table III shows the histopathologic and serologic findings of 9 cases. The histopathologic grades showed varying degrees; however, lymphocytes infiltrated mainly around acinar cells and showed a pattern of benign lymphoepithelial lesion (essential requirement 2). This infiltration pattern of lymphocytes was different from SS, which shows mainly periductal infiltration.

The IgG test was available in 8 cases, and all of them showed high IgG levels. The IgG4 test could be performed only 4 cases; however, they also all showed high IgG4 levels (IgG4 requirement 1). On the other hand, all cases were negative for antiSS-A and antiSS-B antibodies (other condition 2). The results of the IgA and IgM tests were all within normal limits.

Sonographic findings

Six of 9 cases showed normal parotid glands on the sonograms (Table IV; Fig. 2, *A*). Three other cases showed a slight change in parotid glands, which showed multiple hypoechoic areas, either unilaterally or bilaterally; however, they were not necessarily related to the swelling of the glands (Fig. 2, *B*). These hypoechoic areas were observed in normal parotid parenchyma without reduction of echo intensity level and heterogeneity.

On the other hand, all submandibular glands with swelling (8 cases) showed nodal areas on the sonograms (Table IV). They were hypoechoic areas with relatively high vascularization, and bulged from the normal surface of the submandibular glands (Fig. 3, *A* and *B*). Parenchyma of the submandibular glands around nodal areas showed homogeneity and a normal echo intensity level. Such areas were observed bilaterally on 7 of these 8 cases, although the degree of bulge varied from one case to the next (Fig. 3, *C*). In the remaining case, the nodal area was seen unilaterally, because the submandibular gland on the other side had

Table IV. Sonographic findings

	Parotid glands		*Submandibular glands*	
Case no.	*Swelling*	*Sonographic findings*	*Swelling*	*Sonographic findings*
1	−	WNL	−	Bi-SC
2	−	WNL	+	Bi-NA
3	−	WNL	+	Bi-NA
4	−	WNL	+	Uni-NA*
5	+	Uni-SC	+	Bi-NA
6	−	Uni-SC	+	Bi-NA
7	−	Bi-SC	+	Bi-NA
8	−	WNL	+	Bi-NA
9	+	WNL	+	Bi-NA

WNL, Within normal limits; *Uni*, unilateral; *Bi*, bilateral; *SC*, slight change; *NA*, nodal area(s).
*The submandibular gland on the other side had been removed on suspicion of a tumor.

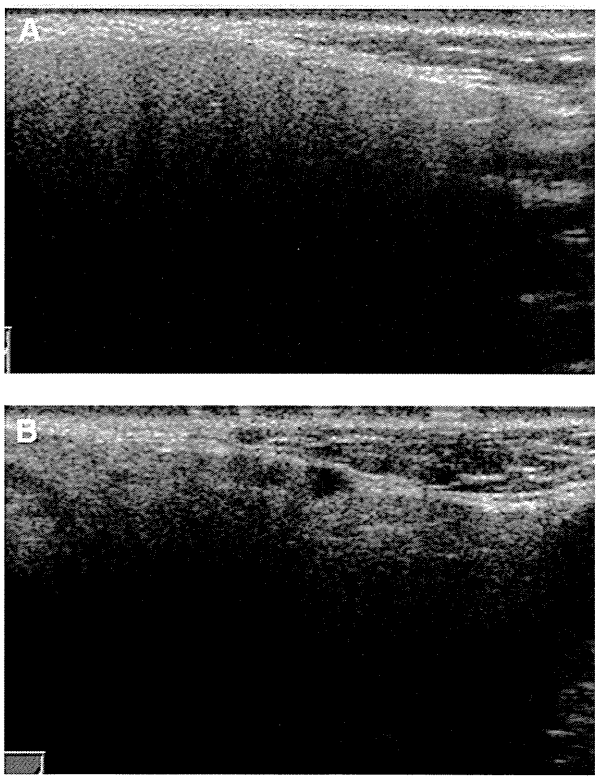


Fig. 2. **(A)** Normal parotid gland sonogram seen in Mikulicz disease (scanned parallel to the retromandibular plane, left: superior, right: inferior). Internal echoes are homogeneous. **(B)** Parotid gland sonogram shows slight change in some cases. Multiple hypoechoic areas are observed in parotid parenchyma with normal echo intensity level.

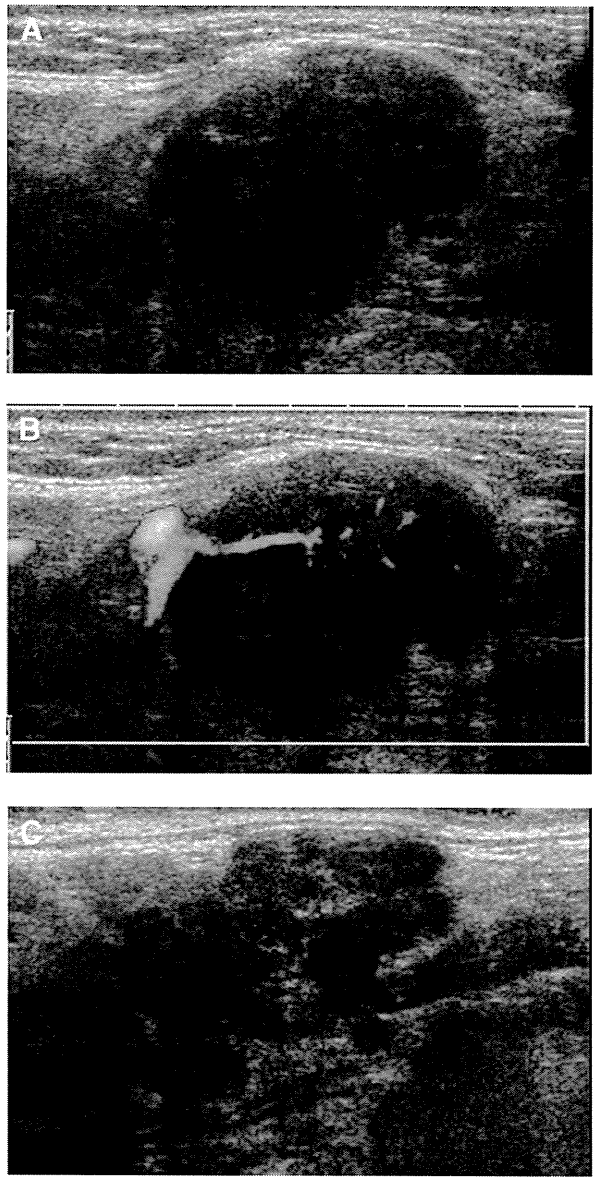


Fig. 3. **(A)** Nodal area in submandibular gland (scanned parallel to the submandibular plane, left: posterior, right: anterior). It is a hypoechoic area bulging from normal surface of the submandibular gland. Note the adjacent submandibular gland parenchyma show normal echo intensity level and homogeneity. **(B)** Doppler mode of the same case as Fig. 3A. The nodal area shows relatively high vascularization. **(C)** Nodal areas show a variety in size and in degree of bulge from case to the next. This case shows smaller nodal area than the case seen in Fig. 3A, 3B (scanned same as Fig. 3A).

been removed owing to suspicion of a tumor. Thus, all submandibular glands with swelling showed bilateral nodal areas on the sonograms. Even 1 case without submandibular gland swelling showed slight change bilaterally on the sonograms.

When we defined the cases with decreased saliva flow in both gum and Saxon tests as cases of objective dryness of mouth, cases 4, 8, and 9 fell into the defi-

Table V. Sialographic findings

	Parotid glands		Submandibular glands	
Case no.	Swelling	Sialographic findings	Swelling	Sialographic findings
1	−	WNL	−	ND
2	−	WNL	+	GD
3	−	DD	+	ND
4	−	WNL	+	ND
5	+	WNL	+	GD
6	−	WNL	+	GD
7	−	WNL	+	ND
8	−	WNL	+	GD
9	+	WNL	+	GD

WNL, Within normal limits; *DD*, ductal dilation; *ND*, not done; *GD*, glandular defect.

nition. Sonograms of these cases showed findings similar to those cases without objective dryness.

Sialographic findings

Excepting 1 case of ductal dilation, 8 of 9 cases showed normal parotid gland sialograms (Table V). No cases showed punctate or globular patterns, which is in contrast to SS (other condition 3; Fig. 4). Two cases with swelling of the glands showed also normal sialograms.

On the other hand, defects in glandular images were observed on all submandibular gland sialograms in accordance with the nodal areas on the sonograms, although submandibular gland sialography could be performed on only 5 of 9 cases (Fig. 5).

Findings on other imaging modalities

Table VI shows the findings of nodal areas on submandibular gland sonography on other imaging modalities. A CT examination was performed in 6 cases. Nodal areas on the sonograms could not be differentiated from adjacent normal glandular tissues. Those areas were demonstrated as low-signal-intensity masses on a T2-weighted MRI in 2 cases. In the other 3 cases, however, they could not be differentiated from adjacent normal glandular tissues, even on MRI. On gallium scintigraphy, 2 cases showed abnormal uptake in accordance with those areas, and 1 case did not show particular findings.

Follow-up examinations

One case was mainly followed up by MRI, and CT was performed in 2 other cases, because these cases were complicated by autoimmune pancreatitis. Sonography was performed in 4 of the 9 cases as a follow-up examination of the persistent salivary gland swelling. Submandibular gland nodal areas in 2 cases showed reduction in size, and 1 case showed no interval change. One case, in which glucocorticoid administration was refused, showed deterioration in the condition of the salivary gland. The bilateral parotid gland showed multiple hypoechoic areas in this case at the time of fol-

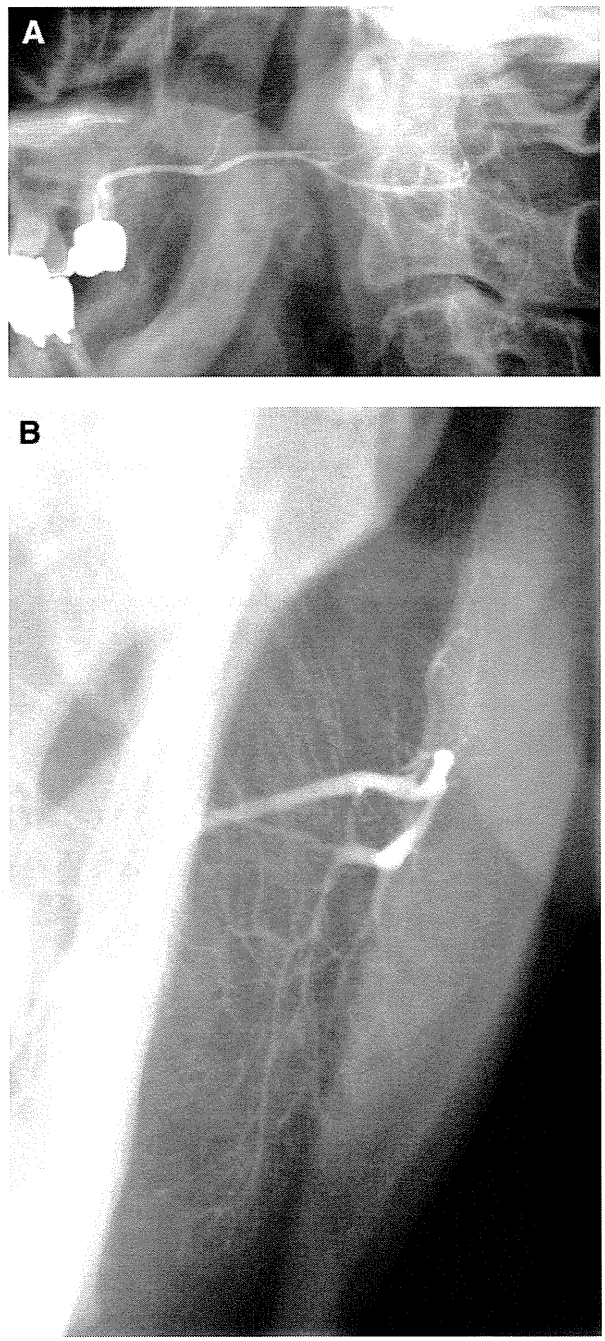
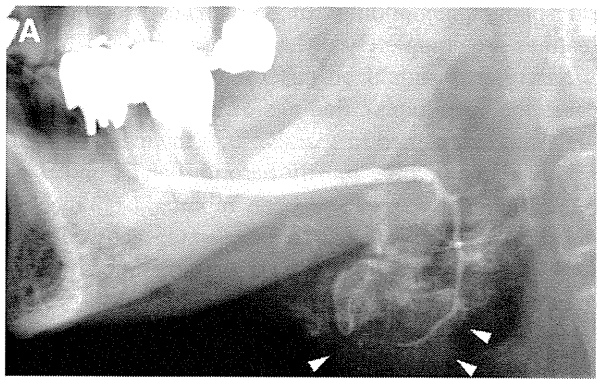


Fig. 4. **(A)** Parotid gland sialogram shows normal findings without punctate or globular pattern seen in Sjögren syndrome (lateral projection). **(B)** Same case as Fig. 4A in postero-anterior projection.



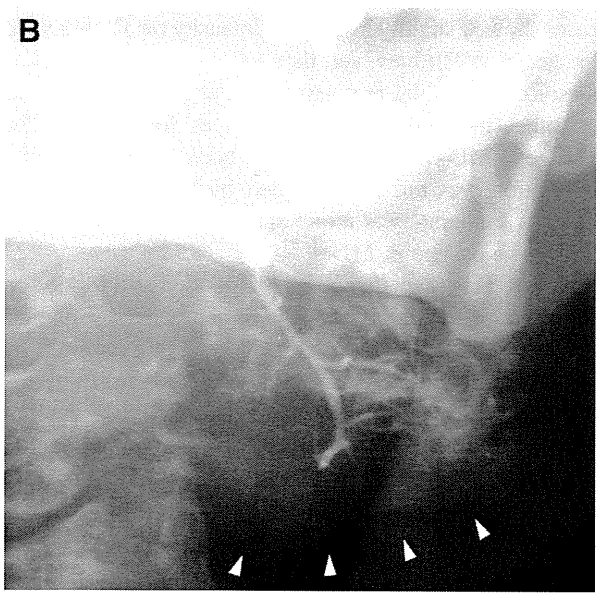


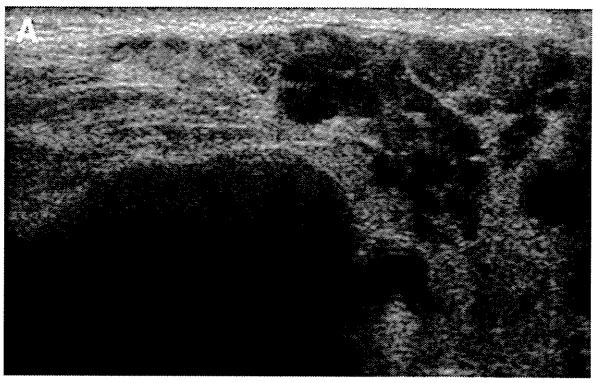
Fig. 5. **(A)** Submandibular gland sialogram shows parenchymal defect (arrowheads) in accordance with the nodal area on the sonogram (lateral projection). **(B)** Same case as Fig. 5A in oblique projection. Note parenchymal defect (arrowheads).

Table VI. Sonographic nodal areas on other imaging modalities

Case no.	*CT*	*MRI*	*RI (gallium)*
2	Unclear	Unclear	ND
3	ND	Low in T2	ND
4	ND	Low in T2	ND
5	Unclear	Unclear	ND
6	Unclear	ND	Abnormal uptake
7	Unclear	ND	Abnormal uptake
8	Unclear	Unclear	ND
9	Unclear	ND	Unclear

CT, Computerized tomography; *MRI*, magnetic resonance imaging; *RI*, radionuclide imaging (scintigraphy); *ND*, not done.

low-up (Fig. 6), although nodal change in the submandibular gland showed no interval change. Unlike SS, these multiple hypoechoic areas were surrounded by



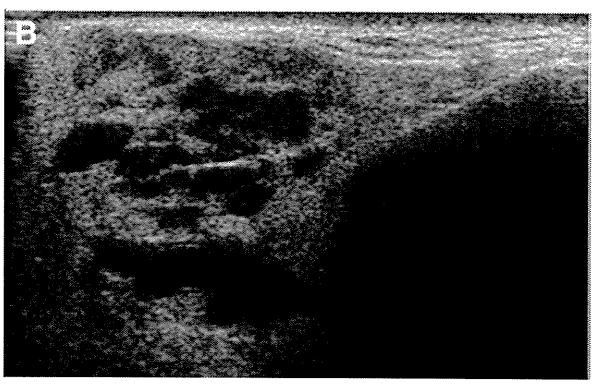


Fig. 6. **(A)** Parotid gland sonogram which shows deterioration in the condition in a follow-up period. Multiple hypoechoic areas are distinct, however, adjacent parotid parenchyma are still within normal echo intensity level (scanned parallel to the Frankfort-Horizonral plane, left: anterior, right: posterior). **(B)** Same case as Fig. 6A (scanned parallel to the retromandibular plane, left: superior, right: inferior).

normal parotid gland parenchyma. Parotid gland parenchyma in SS are generally heterogeneous and show decrease in echo intensity level.[17]

DISCUSSION

Unanimous criteria of MD have not been defined so far, although a new concept of MD has been recently discussed. In the present criteria, we emphasized a histopathologic pattern of lymphocytes different from SS. Lymphocytes infiltrate mainly around acinar cells in MD, whereas SS shows mainly periductal infiltration. Yamamoto et al.[14] showed figures in which the difference in the amount of plasmacytes with IgG4 between MD and SS could be seen. These figures also showed the difference of lymphocyte infiltration patterns. These infiltration patterns can explain why cases of MD respond well to glucocorticoid administration. Ducts in MD are not severely affected; therefore, regeneration of the acinar cells can be easily induced.

In other reports, persistent bilateral swelling in >2 of the lacrimal, parotid, or submandibular glands was the

requirement for an ailment to be considered to be MD. However, we considered an ailment to be MD if only 1 of these glands experienced persistent bilateral swelling. Although 3 of the 9 cases showed only 1 pair of swollen glands, they showed typical findings of MD such as high IgG and IgG4 values and negative results on both antiSS-A and antiSS-B antibody tests.[19]

It is still controversial whether or not cases showing features of both MD and SS should be classified as MD. For example, Yamamoto et al.[20] reported a case of MD with autoimmune pancreatitis, which also met the criteria of SS. To clarify the imaging characteristics of MD more clearly, we excluded the cases showing punctuate or globular pattern on sialograms from this study.

We could not find enough references which showed change dominant in submandibular glands rather than in parotid glands. Tsubota et al.[11] used both lacrimal and parotid gland swelling as criteria of MD, whereas the case reported by Miyake et al.[19] showed only submandibular gland swelling. A case of Yamamoto et al.[20] and a case of Shimoyama et al.[21] suffered from lacrimal and submandibular gland swelling. Lee et al.[22] showed 2 cases: In 1 case lacrimal and parotid gland swelling were observed, and in the other case lacrimal, parotid, and submandibular glands were swollen. Thus, dominance in change in submandibular glands was not clearly demonstrated. However, a nodal area in submandibular glands was removed by an operation in 1 of the 2 cases of Lee et al.[22] and in 1 of the present 9 cases. One case in the present study, in which glucocorticoid administration was refused, showed deterioration of the condition of the salivary glands. The bilateral submandibular glands in this case showed nodal change with no interval change. Whereas the parotid glands showed deterioration and no nodal formation could be seen. These facts suggested that the change in submandibular glands was more dominant than that in parotid glands.

Nodal areas on sonograms in MD resembled Küttner tumor (chronic sclerosing sialadenitis) and other salivary gland tumors; however, they appeared bilaterally in MD. Furthermore, the areas in MD showed relatively high vascularization compared with Küttner tumor and other salivary gland tumors.

Salivary glands affected with SS show characteristic sonographic findings with hypoechoic areas delineated by hyperechoic lines or spots.[17] The change involves the whole gland; therefore, parenchyma are heterogeneous and show a decrease in echo intensity level. Submandibular glands show obscuration of gland configuration but no nodal areas.[17] Parotid glands rarely reveal nodal areas. When parotid glands show nodal areas, however, they are always accompanied by typical sonographic findings of SS.[23] These sonographic features are completely different from those of MD.

Sarcoidosis should be differentiated from MD, because the sonographic findings of both diseases can be very similar in parotid glands.[24,25] However, sarcoidosis tends to involve mostly parotid glands, and if no bilateral nodal areas have been reported,[26,27] these 2 diseases can be differentiated by sonography. Therefore, sonographic examination is recommended in making an imaging diagnosis.

These nodal areas on sonograms were not clearly demonstrated on other modalities. CT[14,21,22] and MRI[20,21] could show only glandular swelling. Some of the present cases showed slightly low signal intensity on T2-weighted images on MRI; however, they were not significant. The reasons these nodal areas were not clearly demonstrated on other modalities can be given as follows: 1) The change in MD was not neoplastic; and/or 2) lymphocytes infiltration was not accompanied by fibrosis or edema. Besides sonography, gallium scintigraphy seemed sensitive to the change in MD.[19-21]

Sonography can also be recommended as a follow-up examination of persistent swelling in salivary glands. Change in salivary glands can be easily examined by sonography without using ionizing radiation. In the present study, because the objective dryness was not related to the swelling, and because the other imaging modalities were not sensitive to the nodal areas, we could conclude that it is effective to observe the glands by sonography to see if they are back on the recovery track or not.

In conclusion, salivary glands affected by MD showed bilateral nodal change in submandibular glands at high rates. Nodal changes were detected as hypoechoic areas with relatively high vascularization by sonography and a parenchymal defect on sialograms. Those changes were completely different from previously reported sonographic characteristics of SS. To detect and also as a follow-up examination of these changes, sonography is indicated as the best imaging modality.

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Enteral Tube Feeding Alters the Oral Indigenous Microbiota in Elderly Adults▽†

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Enteral tube feeding is widely used to maintain nutrition for elderly adults with eating difficulties, but its long-term use alters the environment of the oral ecosystem. This study characterized the tongue microbiota of tube-fed elderly adults by analyzing the 16S rRNA gene. The terminal restriction fragment length polymorphism (T-RFLP) profiles of 44 tube-fed subjects were compared with those of 54 subjects fed orally (average age, 86.4 ± 6.9 years). Bar-coded pyrosequencing data were also obtained for a subset of the subjects from each group (15 tube-fed subjects and 16 subjects fed orally). The T-RFLP profiles demonstrated that the microbiota of the tube-fed subjects was distinct from that of the subjects fed orally (permutational multivariate analysis of variance [perMANOVA], $P < 0.001$). The pyrosequencing data revealed that 22 bacterial genera, including *Corynebacterium*, *Peptostreptococcus*, and *Fusobacterium*, were significantly more predominant in tube-fed subjects, whereas the dominant genera in the subjects fed orally, such as *Streptococcus* and *Veillonella*, were present in much lower proportions. Opportunistic pathogens rarely detected in the normal oral microbiota, such as *Corynebacterium striatum* and *Streptococcus agalactiae*, were often found in high proportions in tube-fed subjects. The oral indigenous microbiota is disrupted by the use of enteral feeding, allowing health-threatening bacteria to thrive.

Enteral tube feeding is widely used to maintain nutrition in patients with a functional gastrointestinal tract but inadequate oral intake. It is frequently used to address eating problems in frail older adults, especially those with dementia. In the United States, approximately one-third of nursing home residents with advanced dementia are tube-fed (22). Nevertheless, tube feeding in the demented elderly remains controversial. Several studies have shown that tube feeding is associated with poor survival (1, 16, 23) and an increased risk of developing pneumonia (12, 15, 28).

The oral indigenous microbiota exists in a state of balance with the host (7), but the long-term use of tube feeding alters the intraoral conditions. The absence of food passage results in an absence of mechanical clearance within the mouth and reduces saliva secretion. The mucosal surfaces often dry out, and dried sputum adheres to the palate. These ecological changes should affect the bacterial population of the indigenous microbiota. Aspiration pneumonia is a major cause of death in tube-fed elderly patients, and it mainly involves oral contents (21). Unexpected bacteria may thrive in the disused oral cavity and threaten the lives of these patients.

Some well-known respiratory pathogenic bacteria, such as *Pseudomonas aeruginosa*, are isolated more frequently from the oropharynges of tube-fed older adults than from adults fed orally (17, 18, 30). However, the overall composition of the oral microbiota remains poorly understood due to its complexity. In addition, opportunistic bacteria could be critical etiologic agents in compromised elderly adults. Recently, we found that the global composition of the tongue microbiota is associated with the risk of pneumonia-related health problems in older adults by using terminal restriction fragment length polymorphism (T-RFLP) analysis of the 16S rRNA gene, which is a culture-independent community-fingerprinting approach (31). The current study examined the rough composition of the tongue microbiota of bedridden elderly adults by using T-RFLP and in a subset of the subjects in more detail by using bar-coded pyrosequencing. In this study, the microbiota of subjects fed enterally was compared comprehensively with that of subjects fed orally to characterize the oral indigenous microbiota of tube-fed elderly adults.

MATERIALS AND METHODS

Study population. The subjects were a subgroup of the population analyzed in our previous study (31). Of the 11 hospitals or nursing homes in the previous study, we selected two hospitals and one nursing home that had sufficient numbers of both tube-fed and orally fed bedridden patients; this study enrolled 98 bedridden elderly residents aged 65 and over (12 men and 86 women; mean age, 86.4 ± 6.9 years). Forty-four subjects were fed with enteral tubes (31 with percutaneous endoscopic gastrostomy [PEG] tubes and 13 with nasogastric [NG] tubes), and 54 were fed orally. The ethics committee of the Kyushu University Faculty of Dental Science approved this study and the procedure for obtaining informed consent. The clinical condition of each subject was evaluated using previously described criteria (31).

Sample preparation. Sample collection and DNA extraction from each sample were performed in our previous study (31). Tongue-coating samples were collected by scraping from the base to the tip of the tongue dorsum with a sterile plastic spatula (Muddler; Nihon Dixie, Yokohama, Japan), and DNA was extracted from each sample.

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T-RFLP analysis. All 98 samples were examined using T-RFLP analysis. From each sample, the internal regions of 16S rRNA genes were amplified using the universal forward primer 8F (5′-AGA GTT TGA TYM TGG CTC AG-3′) labeled at the 5′ end with 6-carboxyfluorescein (6-FAM) and the universal reverse primer 806R (5′-GGA CTA CCR GGG TAT CTA A-3′) labeled at the 5′ end with hexachlorofluorescein (HEX). PCR was performed using the KOD DNA polymerase (Toyobo, Osaka, Japan) and cycling conditions of 98°C for 2 min followed by 30 cycles of 98°C for 15 s, 60°C for 20 s, and 75°C for 30 s. The 16S rRNA gene amplicons were gel purified using a Wizard SV gel and PCR purification kit (Promega, Madison, WI). Digestion with the restriction enzyme HaeIII and electrophoresis were performed as described previously (31). The 98 T-RFLP profiles containing the electropherogram data were aligned using two different fluorescent dyes (6-FAM and HEX) per subject. The aligned T-RFLP profiles, excluding those terminal restriction fragments (TRFs) detected in fewer than 10% of the subjects, were subjected to principal-component analysis (PCA) and displayed as a PCA diagram. PCA was performed using R 2.10.0 (26). Candidate bacterial species corresponding to important TRFs were selected based on their sizes from 755 oral bacterial 16S rRNA gene sequences (HOMD 16S rRNA RefSeq version 10.1) deposited in the Human Oral Microbiome Database (9). The matching window was set to a molecular weight (MW) of ±660.

Bar-coded pyrosequencing analysis. Pyrosequencing of the 16S rRNA gene was performed for 15 subjects fed by tube and 16 subjects fed orally; subjects were selected randomly from the 25 PEG tube-fed subjects and 42 subjects fed orally who had not used antibiotics in the preceding month. For each extracted DNA sample, we reamplified the 16S rRNA gene using 806R with the 454 Life Sciences adaptor B sequence (5′-CCT ATC CCC TGT GTG CCT TGG CAG TCT CAG-3′) and 8F with the 454 Life Sciences adaptor A and subject-specific 6-base bar code sequences (5′-CCA TCT CAT CCC TGC GTG TCT CCG ACT CAG NNN NNN-3′). The PCR amplification was performed under the same conditions as for the T-RFLP analysis. The proper size of amplicons was confirmed by agarose gel electrophoresis, and amplicons were gel purified using a Wizard SV Gel and PCR clean-up system (Promega) according to the manufacturer's instructions. The DNA concentration and quality were assessed using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE), and equal amounts of DNA from 31 subjects were pooled together. One microliter of the mixture was electrophoresed on an agarose gel to reconfirm the proper size of the amplicons. Pyrosequencing was carried out on a 454 Life Sciences genome sequencer FLX instrument (Roche, Basel, Switzerland) at the Dragon Genomics Center of Takara Bio (Yokkaichi, Japan) and was used to determine the 16S rRNA gene sequences containing the hypervariable regions V1 and V2.

Informatics analysis. The pyrosequencing reads were processed according to the procedure described by Costello et al. (6), with some modification. Sequences were excluded from the analysis using a script written in PHP if they were shorter than 240 bases or had an average quality score of <25 and were subsequently removed using a script written in R if they did not include the correct primer sequence, had a homopolymer run of >6 nucleotides (nt), or contained three or more ambiguous characters. The remaining sequences were assigned to each subject by examining the six-base bar code sequence. Similar sequences were clustered into operational taxonomic units (OTUs) using the complete-linkage clustering tool of the RDP pyrosequencing pipeline (5) at a distance cutoff of 0.03, and representative sequences of each cluster were selected using the Dereplicate request function. The representative sequences from each OTU were aligned using PyNAST (4) and the Greengenes database (8) using a minimum percent identity of 75%. Chimeras were removed from the representative set on the basis of identification as chimeric via Chimera Slayer (13) and verification that the putative chimera appeared in only one sample. After chimera elimination, a relaxed neighbor-joining tree was built using FastTree (25). To determine the dissimilarity between any pair of bacterial communities, we used the UniFrac metric (20) calculated by Fast UniFrac (14). UniFrac distances are based on the fraction of branch length shared between two communities within a phylogenetic tree constructed from all communities being compared. The similarity relationship assessed using the unweighted UniFrac metric was represented in a principal-coordinate analysis (PCoA) plot drawn by R. The taxonomy of representative sequences was determined using the RDP classifier with a minimum support threshold of 60% and the RDP taxonomic nomenclature (down to the genus level). To detect the OTUs characteristically detected in the tube-fed subjects, we considered only OTUs containing at least 100 sequences. For each representative sequence of the OTUs detected in significantly higher proportions in the tube-fed group than in the orally fed group, the nearest-neighbor species with over 98% identity were first searched using BLAST against 755 oral bacterial 16S rRNA gene sequences (HOMD 16S rRNA RefSeq Version 10.1) deposited in the Human Oral Microbiome Database (9). Sequences with no hits were further compared against a local database comprising 81,679 nonchimeric 16S rRNA gene sequences of "ncbi_tax_string" not deposited as "environmental samples" in the Greengenes database (8).

TABLE 1. Baseline characteristics of bedridden elderly adults fed orally or by tube

Characteristic	Value[a] for: Tube-fed patients (n = 44)	Orally fed patients (n = 54)	P value
General conditions			
Age, yr	85.1 ± 7.5	87.4 ± 6.3	0.10
Female sex, no. (%)	35 (79.5)	51 (94.4)	0.03
Institution, no. (%)			0.06
Hospital A	27 (61.3)	40 (74.0)	
Hospital B	14 (21.6)	7 (12.9)	
Nursing home A	3 (6.8)	7 (12.9)	
Dementia, no. (%)			<0.001
Mild	5 (11.3)	29 (53.7)	
Severe	39 (88.6)	25 (46.2)	
Coexisting conditions, no. (%)			
Diabetes mellitus	5 (11.3)	5 (9.2)	0.74
Stroke	38 (86.3)	37 (68.5)	0.05
Cancer	5 (11.3)	9 (16.6)	0.56
Chronic gastroenteritis	3 (6.8)	4 (7.4)	1.00
Cardiovascular disease	18 (40.9)	24 (44.4)	0.83
Kidney disease	2 (4.5)	3 (5.5)	1.00
Liver disease	6 (13.6)	8 (14.8)	1.00
Oral conditions			
No. of natural teeth	8.4 ± 9.3	7.8 ± 7.6	0.70
No. of decayed teeth	1.7 ± 3.3	2.1 ± 3.6	0.53
Denture use, no. (%)	0 (0)	16 (29.6)	<0.001
Amt of tongue coating, no. (%)			0.004
No or slight	15 (34.0)	35 (64.8)	
Moderate or much	29 (65.9)	19 (35.1)	
Tongue moisture (mm)			1.00
≥5.0	13 (29.5)	13 (24.0)	
1.0–4.9	14 (31.8)	22 (40.7)	
<1.0	17 (38.6)	19 (35.1)	

[a] Values with errors are means ± standard deviations.

Statistical analysis. All statistical analyses were conducted with R. Fisher's exact test was conducted to look for differences by sex, institution, severity of dementia, coexisting conditions, denture use, amount of tongue coating, and tongue moisture. Student's t test was performed to compare age and the numbers of teeth and decayed teeth. Wilcoxon's signed-rank test was performed to compare the relative abundance of bacteria. Permutational multivariate analysis of variance (perMANOVA) with the function adonis in the vegan package was performed to test for differences in bacterial community structure among groups of samples. Statistical significance was set at a P value of <0.05.

RESULTS

Of the 98 bedridden elderly persons in this study, 44 were fed via enteral tubes (31 by PEG tubes and 13 by NG tubes), and 54 were fed orally. Table 1 summarizes the general and oral conditions of the subjects in each group. Although significantly more men and severely demented persons were included in the tube-fed group, no statistically significant differences were observed for the other general conditions. The amount of tongue coating was significantly greater in the tube-

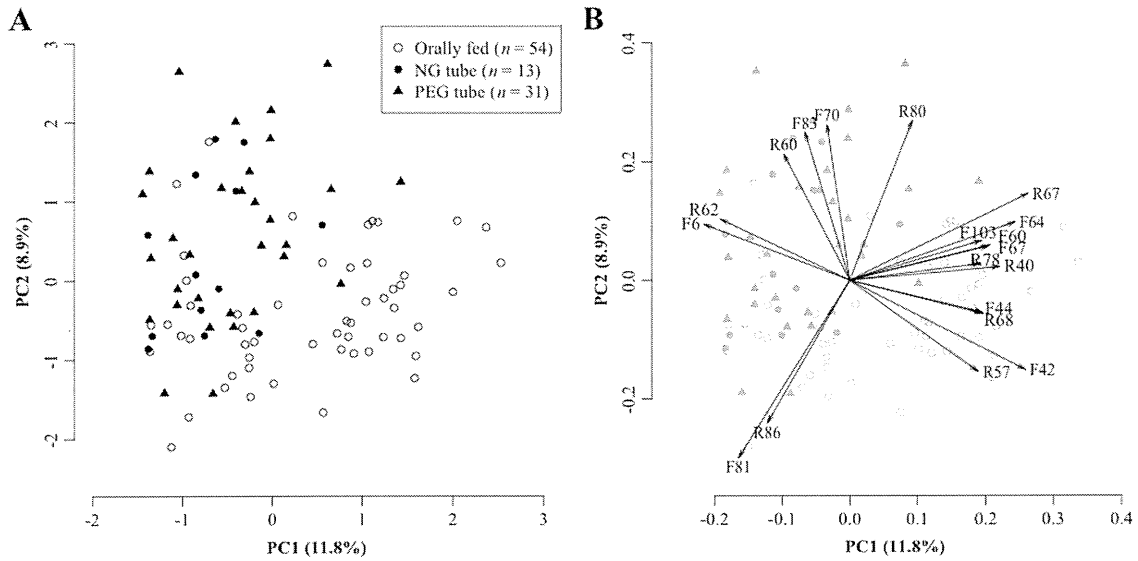

FIG. 1. (A) Principal-component analysis (PCA) diagram showing the similarity relationships among the 98 T-RFLP profiles. The T-RFLP profile of each subject is plotted according to feeding mode: orally (○), by percutaneous endoscopy gastrostomy (PEG) tube (▲), and by nasogastric (NG) tube (●). These two components explain 20.7% of the variance. (B) Loading plot of the first two principal components. Only 19 TRFs with large factor loading (>0.5 in absolute value) on the first or second principal component were selected, and these are indicated by arrows.

fed group than in the group fed orally. No denture users were included in the tube-fed group.

The tongue microbiota compositions of all 98 subjects were compared based on the T-RFLP profiles of the 16S rRNA gene. The overall profiles contained 235 distinct peaks (TRFs), 121 TRFs (F1 to F121) in the 6-FAM profiles and 114 TRFs (R1 to R114) in the HEX profiles. To visualize the similarity of T-RFLP profiles, they were plotted in a PCA diagram of the first principal component (PC1) and the second principal component (PC2) using different dots to represent each feeding mode (Fig. 1A). These two components explained only 20.7% of the total variation. This low value represents the large diversity in the microbiota structures of bedridden elderly subjects. Their diverse T-RFLP patterns containing various uncommon TRFs might not be well explained by using only two virtual factors. The performance of two-factorial PCA to explain overall microbial community variability is limited. Nevertheless, the diagram of these two primary principal components showed that the T-RFLP profiles of both PEG tube-fed subjects and NG tube-fed subjects differed greatly from those of subjects fed orally. The differences between the two groups were confirmed statistically using perMANOVA ($P < 0.001$). No significant difference was observed between PEG and NG tube feeding.

The loading plot of the first two principal components gave us some phylogenetic information on the microbiota of tube-fed subjects (Fig. 1B). TRFs with a large (>0.5 in absolute value) factor loading in the negative direction of PC1 were F6 and R62. Based on the fragment size, *Corynebacterium* or *Propionibacterium* species were selected from the oral bacterial database as candidate bacterial species corresponding to these TRFs (see Table S1 in the supplemental material). Conversely, 11 TRFs (R67, F42, F64, R40, F60, F67, R68, F44, F103, R78, and R57) had a large positive loading on PC1; they corresponded to other bacterial species, including *Prevotella* and *Veillonella*. Two TRFs with high loading in the negative direction of PC2 (F81 and R86) corresponded to *Streptococcus* or *Bacillus* species, while bacteria of the genus *Fusobacterium* and family *Peptostreptococcaceae* were assigned to four TRFs (R80, F70, F83, and R60) with large positive loading on PC2. Tube-fed subjects were localized in the negative direction of PC1 and the positive direction of PC2 (upper left area in the diagram), suggesting that their microbiotas contain lower proportions of common oral bacteria such as *Streptococcus*, *Veillonella*, and *Prevotella* and higher proportions of other bacterial species, including *Corynebacterium* and *Fusobacterium*, than those of orally fed subjects.

To obtain more-detailed phylogenetic information, barcoded pyrosequencing analysis was performed for 15 PEG tube-fed and 16 orally fed subjects. We determined 131,888 sequences, and 103,391 bacterial 16S rRNA gene sequences with an average length of 358 ± 71 bases passed quality control (Table 2). The sequences were assigned to 3,118 species-level OTUs using a cutoff distance of 0.03. The PCoA plot based on UniFrac, which is a phylogeny-based metric (20), also revealed that the overall microbiota composition in the tube-fed subjects was distinct from that in those fed orally (Fig. 2).

Although the microbiota diversity was confined largely to five phyla (*Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, *Firmicutes*, and *Proteobacteria*) in both groups, the relative proportions of these phyla varied greatly between the two groups. The tube-fed subjects had a significantly higher proportion of *Actinobacteria* and a lower proportion of *Firmicutes* than those fed orally (Fig. 3). In addition, the relative abundances of three minor phyla, *Synergistetes*, *Tenericutes*, and SR1, were significantly greater in the tube-fed group.

At the genus level, dominant bacterial genera commonly detected in the orally fed subjects, such as *Veillonella* and *Streptococcus*, were much less predominant in the tube-fed subjects (Fig. 4). Conversely, 22 minority bacterial genera in

TABLE 2. Summary of pyrosequencing analysis

Group (*n*) and subject	No. of reads
Tube fed (15)	
T1	2,446
T2	4,376
T3	898
T4	6,139
T5	3,098
T6	2,454
T7	4,839
T8	1,898
T9	7,061
T10	1,707
T11	1,717
T12	3,689
T13	1,579
T14	954
T15	4,987
Avg ± SD	3,189 ± 1,913
Orally fed (16)	
O1	2,530
O2	2,781
O3	1,347
O4	6,237
O5	2,879
O6	1,896
O7	8,381
O8	3,435
O9	6,986
O10	2,431
O11	1,336
O12	1,468
O13	3,068
O14	1,504
O15	6,490
O16	2,780
Avg ± SD	3,471 ± 2,252

the usual oral cavity, including *Corynebacterium*, *Peptostreptococcus*, and *Fusobacterium*, accounted for markedly higher proportions in the microbiota of tube-fed subjects (Fig. 5). Seven bacteria unclassified at the genus level (family *Flavobacteriaceae*, family *Neisseriaceae*, family *Pasteurellaceae*, family *Synergistaceae*, family Incertae Sedis XI, order *Bacteroidales*, and phylum *Bacteroidetes*) were also more predominant in tube-fed subjects than in subjects fed orally. The genera *Pseudomonas* and *Acinetobacter* were detected only in the tube-fed group (one and three subjects, respectively). *Staphylococcus* was detected in four tube-fed subjects and one orally fed subject. *Klebsiella* was not detected in these subjects.

At the species level, defined as the 3% dissimilarity level, 54 OTUs in the tube-fed group were found in significantly higher proportions than in the orally fed group (see Table S2 in the supplemental material). Eight of these OTUs corresponded to bacterial species rarely detected in the oral cavity, such as *Corynebacterium striatum*, *Streptococcus agalactiae*, and *Streptococcus dysgalactiae*.

DISCUSSION

This study demonstrated that the oral microbiota of tube-fed older adults is distinct from that of those fed orally. Although the microbial composition varied among the subjects fed orally, the difference according to the feeding mode exceeded the interindividual differences (Fig. 1 and 2). Predominant indigenous members such as *Streptococcus* and *Veillonella* were detected in much lower proportions in the tube-fed subjects, whereas as many as 22 bacterial genera, including *Corynebacterium*, occurred in significantly higher proportions than in the orally fed subjects (Fig. 4 and 5). Bacterial species normally uncommon in the oral cavity, such as *C. striatum*, were also found in high proportions in their microbiota. This characteristic microbiota composition was observed in both the PEG and NG tube-fed subjects (Fig. 1), suggesting that it is not derived from biofilm formed on the feeding tube. We postulate that the normal balance of the microbiota is disrupted in the oral cavity when it is not used for eating. The long-term absence of food passage is an extremely abnormal situation for the oral indigenous microbiota. While fluid and carbohydrate supply is stopped, mechanical clearance by mastication drastically decreases. In addition to a reduction in the salivary flow, the biochemical composition of saliva in tube-fed subjects differs from that in orally fed ones (18). These ecological changes would be involved in the disruption of the indigenous microbiota.

No significant increase in typical respiratory pathogens, such as *P. aeruginosa*, was observed in this study, but the species that thrived in their microbiota could threaten the lives of frail elderly adults. The predominant *Corynebacterium* species, especially *C. striatum*, are potentially pathogenic bacteria with the ability to cause nosocomial outbreaks and respiratory colonization (24, 27). Anaerobic bacterial genera such as *Fusobacterium*, *Peptostreptococcus*, *Parvimonas*, and *Porphyromonas* are associated with pulmonary infections, such as pneumonia, lung abscesses, and empyema (2, 3, 11, 34). In contrast to the oral *Streptococcus* species, *S. agalactiae* accounted for high proportions in the microbiota in tube-fed patients, and these

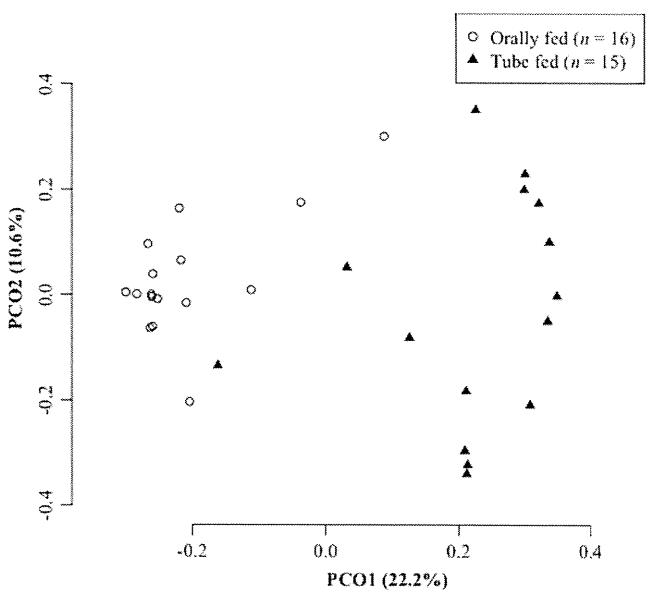

FIG. 2. Principal-coordinate analysis (PCoA) plot showing the similarity relations among the 31 tongue microbiota compositions. Plots were generated using unweighted UniFrac distances. These two components explain 32.8% of the variance.

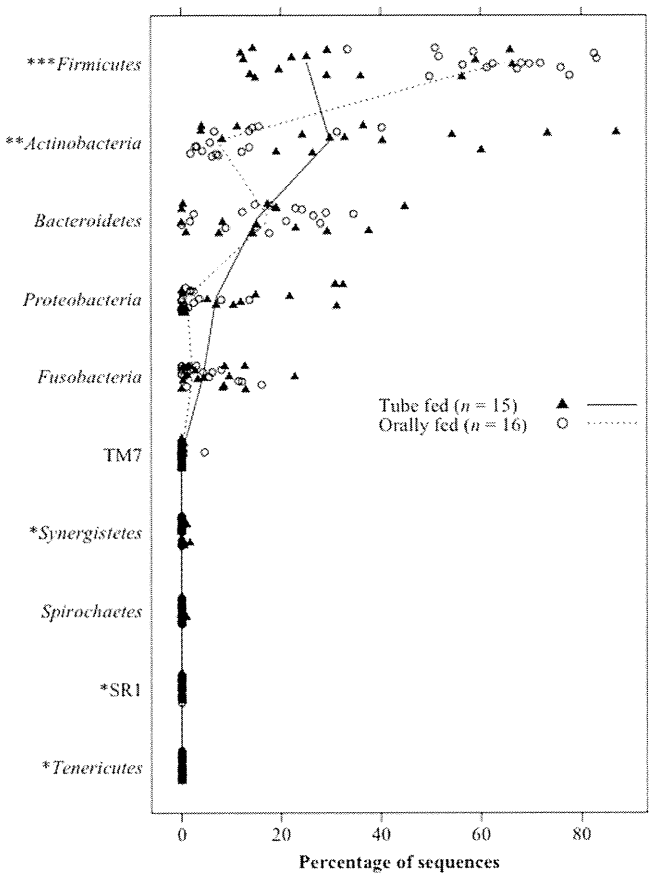

FIG. 3. Relative abundance of each phylum in the tongue microbiota of 31 subjects. The median percentages in the tube-fed and orally fed subjects are represented by solid and broken lines, respectively. *P* values were calculated using Wilcoxon's signed-rank test. *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$.

cause invasive disease in elderly adults (10). Although mealtime aspiration is averted by the use of a feeding tube, elderly adults fed by tube commonly aspirate contaminated oral secretions (12). Therefore, our results imply that tube-fed elderly adults continuously inhale unusual, more virulent bacteria into the lower respiratory tract and lungs. In addition, the disturbed balance of beneficial and detrimental bacteria in the indigenous microbiota, or dysbiosis, has recently attracted attention in regard to the development of mucosal inflammation, including Crohn's disease (19, 29, 32, 35). The oral dysbiosis that occurs with enteral tube feeding could be a health-threatening factor for frail elderly adults.

Although performing a randomized controlled trial would be difficult, the poor outcome of enteral feeding in elderly adults has been reported in several observational studies (1, 12, 15, 16, 23, 28). In addition, in our study, the incidence of pneumonia or fever and mortality in the following 6 months were significantly higher in the tube-fed subjects than in those fed orally, although this may have been due to differences in the baseline conditions of the two groups (data not shown). One should pay careful attention to the bacterial populations in the oral cavity with the use of feeding tubes. While the benefits of oral care in preventing pneumonia in elderly adults are well documented (33, 36), oral care is generally neglected in patients receiving tube feeding due to the erroneous impression that their oral cavities are not used. Rather, our results suggest that tube-fed patients need aggressive oral care to prevent the overgrowth of a disturbed microbiota, even if such care might be ineffective at restoring the normal microbiota.

This study was cross-sectional and thus cannot unequivocally demonstrate that feeding tube placement results in oral dysbiosis. A follow-up study would clarify the environmental trigger of the dysbiosis associated with tube feeding and may lead to the development of a novel approach to prevent the oral dysbiosis in tube feeding.

In the present study, we used two different molecular approaches for microbiota comparison, T-RFLP and bar-coded pyrosequencing. Although T-RFLP is highly effective for rapid comparisons of bacterial communities, it is unsuitable for predicting microbial community structures containing unexpected bacteria. Indeed, the high proportion of *C. striatum* in tube-fed subjects was unable to be predicted by T-RFLP because *C. striatum* is an uncommon bacterium in the oral cavity and thus is not deposited in the database which we used (the TRF size of *C. striatum* corresponded to F6 and R62). In addition, overgrowth of *S. agalactiae* in tube-fed subjects was masked by a decrease in the dominant oral *Streptococcus* species, such as *S. salivarius*, which generates TRFs with the same size (F81 and

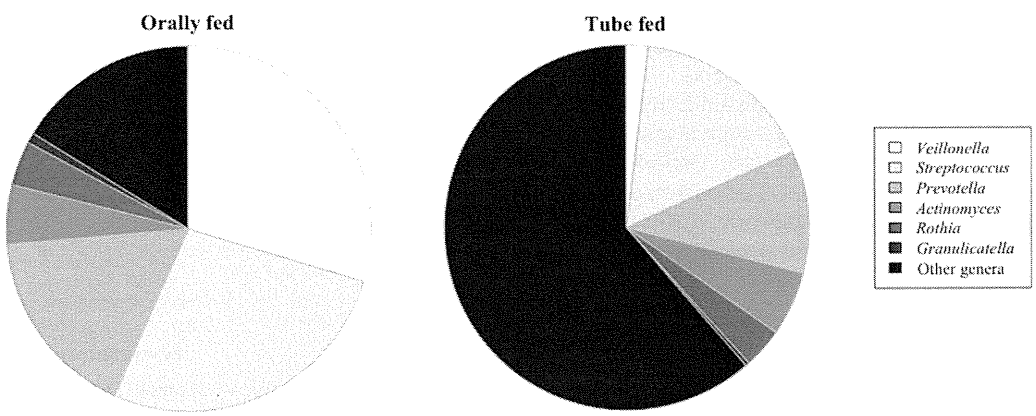

FIG. 4. Mean genus abundances in orally fed and tube-fed subjects. Only six genera commonly detected in the orally fed group (14 of 15 orally fed subjects) are shown.

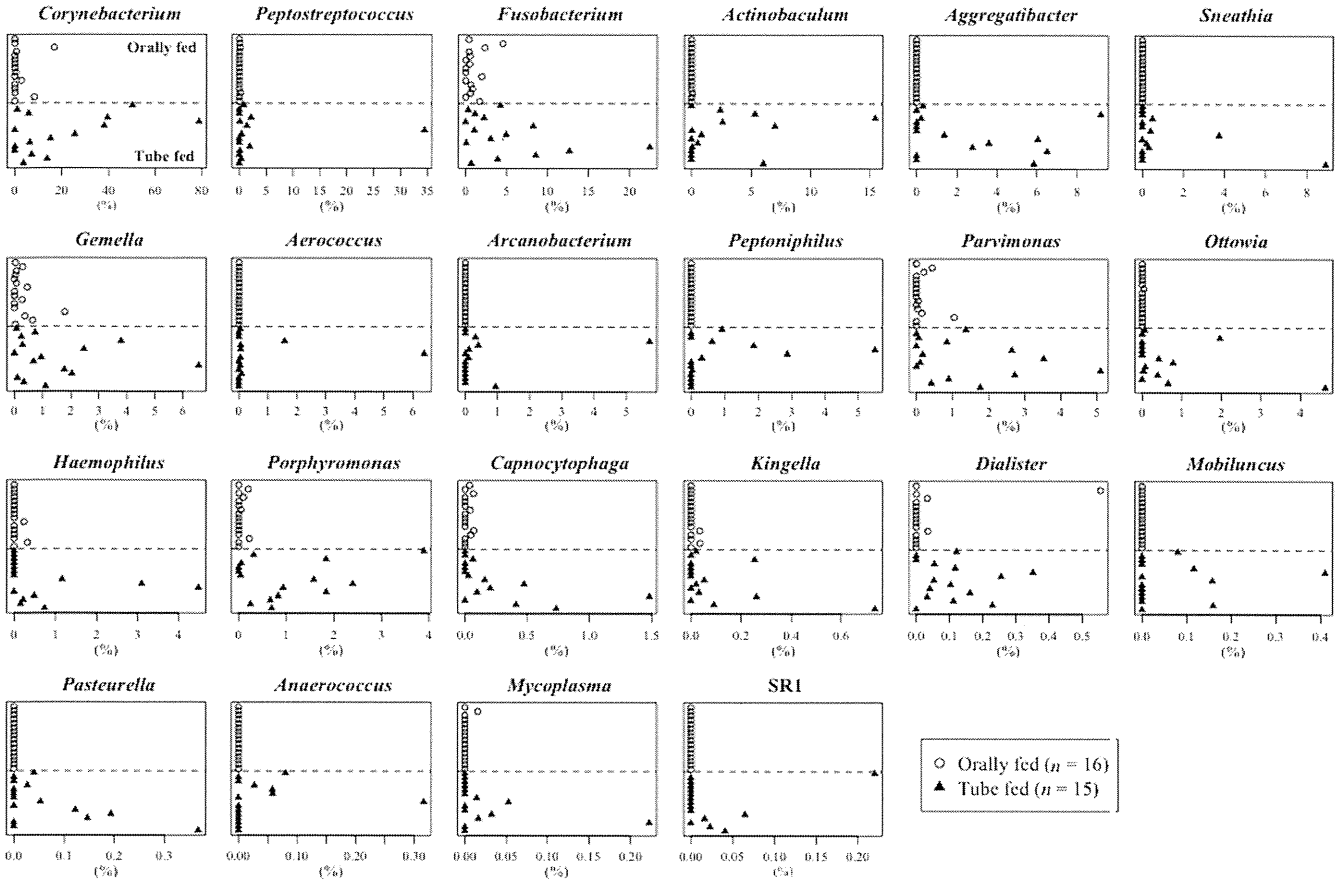

FIG. 5. Relative abundances of 22 bacterial genera that were significantly more predominant in the tube-fed group than in the orally fed group ($P < 0.05$). Statistical differences were calculated using Wilcoxon's signed-rank test.

R86). Nevertheless, the microbiota characteristics of tube-fed subjects predicted from the T-RFLP data were globally consistent with the results of pyrosequencing analysis. Although some limitations exist, T-RFLP is useful for comparisons of oral microbiota, especially in analyses using a large number of samples.

The oral indigenous microbiota is thought to serve as a defensive barrier against the establishment of more pathogenic bacteria (7). Our results clearly demonstrated that the oral indigenous microbiota is disrupted by the use of enteral feeding, allowing health-threatening bacteria to thrive. It is suggested that oral food intake plays an important role not only in nutrition but also in maintenance of a healthy oral indigenous microbiota that acts to prevent exogenous infection.

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特集 口腔内細菌の全身疾患への関わり

2．口腔細菌叢と高齢者の感染症

山下 喜久*1) 柴田 幸江*2) 竹下 徹*3)

全身の抵抗力が低下した高齢者では，肺炎やこれに関連した健康障害が生命を脅かす大きな要因となる。近年，口腔細菌がこのような高齢者の健康問題に大きく影響すると考えられており，高齢者の健康管理の一環として口腔ケアの重要性が認識されるようになってきた。しかし，口腔ケアの指標については今ひとつ明確となっておらず，口腔細菌の病原性についての検査方法の確立が待たれる。本稿ではその方法のひとつとして口腔細菌叢全体としての病原性の評価の試みを紹介し，今後の高齢化社会において歯科医療が果たし得る役割を考察する。

Key Words：口腔細菌叢／Terminal restriction fragment length polymorphism (T-RFLP) 法／高齢者／誤嚥性肺炎

Ⅰ はじめに

世界の多くの先進国では平均寿命の延伸と出生率の低下が普遍的に広がり，高齢化社会への道を歩み続けている。なかでも，わが国では諸外国に例を見ない速さで人口の高齢化が進んでおり，2055年には約2.5人にひとりが65歳以上の高齢者という「超高齢者社会」に突入することが予想されている。さらに，わが国では今後，団塊世代が高齢者群に参入し，さらに平均寿命の延伸と相まって，単に高齢化率が上昇するだけでなく，実質的な高齢者人口が1,400万人近く増加することが大きな問題となっており，高齢者の健康問題はわが国における喫緊の課題となっている。

平成21年（2009年）度の高齢者の死因順位をみると，65～84歳までは，第1位：悪性新生物，第2位：心疾患，第3位：脳血管疾患，第4位：肺炎であるが，85歳以上では肺炎が脳血管疾患を抜いて第3位となり，90歳以上では心疾患に続いて第2位になっている。さらに，肺炎による死亡の88%は75歳以上の高齢者が占めているのが現状であり，高齢者の健康にとって感染症，特に肺炎への対策が重要であることがわかる。

Ⅱ 高齢者の肺炎に対する口腔細菌の病原性

1．高齢者の肺炎の特徴

高齢者の肺炎の特徴は誤嚥性肺炎と言われている。誤嚥には，加齢，脳卒中，全身麻痺，あるいは麻痺などの症状のない脳梗塞などによって咳反射や嚥下反射が低下し，本人の自覚のない状態で咽頭部および口腔内の細菌が唾液とともに肺に流れ込む場合（不顕性誤嚥），また，胃液などの消化液が食べ物とともに食道を逆流して肺に流れ込む場合があり，前者では口腔・咽頭細菌叢を構成する細菌種が誤嚥性肺炎を引き起こす可能性がある。

Infectious diseases related to oral microbiota in elderly
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市中肺炎の起炎菌が口咽・咽頭部に定着する場合には当然そのリスクが増すことが考えられるが，口腔に歯周炎が存在する場合には誤嚥された歯周病菌が肺炎を引き起こすことや，口腔・咽頭部の常在細菌が体力や免疫能の低下した高齢者に対して日和見病原体となって肺炎を引き起こすことも考えられる。

これまでに，口腔細菌が誤嚥性肺炎に関与していることは多くの研究者が報告しており[1)~3)]，さらにYoneyamaら[4)]は，口腔ケアが高齢者の肺炎の発症頻度を減少させることを示している。口腔細菌の中でもいくつかの特徴的な細菌種が誤嚥性肺炎の起炎菌として注目されてきたが，これらの細菌種の病原性は多様であり，また，その菌量や宿主の健康状態によってもその病原性が大きく左右されることが考えられるため，誤嚥性肺炎の明らかな原因菌として特定されるに至っていない。

口腔細菌と誤嚥性肺炎が密接な関係にあることは誰の目にも明らかであるが，全身状態がきわめて不安定な高齢者における誤嚥性肺炎の起炎菌を特定すること自体にそもそも無理があるのかもしれない。全身や口腔の健康状態が幅広い高齢者においては，誤嚥性肺炎と口腔細菌との関連性を調べるにあたって，肺炎に関与する全身疾患や口腔状態などの複数の交絡因子を加味せずに，特定の細菌種と誤嚥性肺炎の関連性を調べてもそこに限界があることは自明の理と言える。

2．高齢者の肺炎と口腔細菌

2001年にTerpenningら[5)]は，外来患者，入院患者ならびに高齢者施設入居者358人について，生活習慣，全身疾患，口腔状態および口腔細菌について調べ，これらのパラメータを加えた多変量ロジスティック回帰分析により，唾液中の*Staphylococcus aureus*，*Porphyromonas gingivalis*および*Streptococcus sobrinus*の存在が誤嚥性肺炎に関係していることを報告している。しかしながら，その際に調べられた細菌種の数は非常に限られており，その根拠も明らかでないことから，この研究結果が意味するところをどのように解釈できるのかについては，今ひとつ定かではない。さらに言えば，高齢者では確かに誤嚥性肺炎が発症する確率は高くなるが，高齢者の肺炎をすべて誤嚥性肺炎と決めつけることはできない。しかも，その診断基準においても嚥下障害の有無が重要なポイントとなるなど，その病態の診断というよりも，むしろ因果関係の推測に基づいた診断と言っても過言ではない。

多くの症例は誤嚥性肺炎の疑いのある症例が誤嚥性肺炎として診断されている可能性も否定できず，多くの研究に示されている誤嚥性肺炎の診断定義を考えると，誤嚥性肺炎との因果関係を調べた研究の多くは，高齢者性肺炎との因果関係を調べたものと考えるほうが適当かもしれない。

我々は口腔の健康を評価する上で口腔細菌叢の全体像を把握することが有用であることを報告している。口腔環境には従来，う蝕や歯周炎の起因菌として報告されてきた細菌種のほかにも莫大な種類と量の細菌種が存在しており，それらの相互関係が個人の口腔疾患に対する感受性に影響を与えていることが考えられる。細菌の16S ribosomal RNA遺伝子のクローニングと，その塩基配列の解析によるクローンライブラリー法のような培養を必要としない分子生物学的手法によって，ヒト口腔からは700種を超える細菌種が同定されている。しかしながら，口腔細菌叢と各個人の口腔の健康との関係を明らかにするためには，多数の被験者からサンプルを採取する必要があり，そのすべてのサンプルをクローンライブラリー法で解析するには多大な費用と時間を要するため，現実的には非常に難しく，口腔細菌叢と口腔の健康の関連性についての研究報告はほとんどなかった。

一方，クローンライブラリー法よりも簡便に環境中の複雑な細菌群集を比較する分子生物学的手法として，Terminal restriction fragment length polymorphism (**T-RFLP**) 法がある。しかしながら，従来のT-RFLP法はDNA断片のサイズの計測精度が低いため，T-RFLP法単独で細菌種を正

T-RFLP (Terminal restriction fragment length polymorphism)

確に同定することは難しく，得られたピークパターンの各ピークに割り振られる細菌種を特定するためにはクローンライブラリー法を併用する必要があった。

そこで我々は，従来のT-RFLP法のサイズ定義を改良することでDNA断片の測定精度を向上させることに成功し，クローンライブラリー法を併用することなくT-RFLP法によるTerminal restriction fragments (**TRFs**) の推定サイズからそのピークに割り振られる細菌種をより正確に特定することを可能にした[6]。この改良T-RFLP法を用いて唾液中の細菌叢を解析した結果，歯周炎患者や口臭の強い患者の唾液中には*Prevotella*属および*Veillonella*属が有意に優勢であることを明らかにしており，本法が臨床評価に有用であることを確認している[7,8]。

Ⅲ 高齢者の健康と口腔細菌叢との関連性を示す研究結果

前述の内容をふまえ，口腔細菌叢と高齢者の肺炎および発熱との関係の解明を試みた[9]。対象者は福岡県大牟田市内の4病院の入院患者と7民間高齢者施設の入所者の中で65歳以上の高齢者343人（平均年齢85.8±7.4，男性85人，女性258人）である。T-RFLP法による舌苔の細菌叢の分析を含めた口腔診査ならびにその他の健康状況調査を行い，その後の肺炎の発症を6カ月間追跡したところ，追跡期間中に35人が肺炎を発症し，その中の11人が死亡した。

一方，追跡期間中に21人が施設を出所し，23人が肺炎を含む何らかの原因で死亡したため，残る299人については診査の前後6カ月，すなわち1年間の発熱日数（37.5℃ 以上が9日以下，あるいは10日以上）を調べ，舌苔中の細菌叢との関連性を調べた。**表1**に，年齢，性ならびに脳卒中やガンなどの基礎疾患を含めた16項目の口腔および全身に関する診査項目の中で肺炎発症の有無および発熱日数と関連していたものを示すが，舌の乾燥，義歯の使用，身体的活動の低下，認知症，嚥下困難，抗生物質の使用が肺炎発症および発熱日数に有意な関連性を示した。

この研究では対象者の多くが病弱な高齢者であったことから刺激唾液が採取しにくいこともあり，歯周病や口臭の研究とは異なり，細菌叢の分析試料として舌苔を使用したが，改良T-RFLP法により解析したところ，被検者の舌苔の細菌叢は**図**に示すようにクラスターA，B，C，Dの4つに分類された。

Cox比例ハザード回帰分析で6カ月の追跡期間中の肺炎頻度のハザード比をこれらのグループ間で比較したところ，**表2**のように，クラスターAに対しクラスターCでハザード比4.0（95%信頼区間1.1～15.1），クラスターDでハザード比4.9（95%信頼区間1.2～21.1）と高値を示し，他の肺炎発症の交絡因子（舌の乾燥，嚥下困難，身体的活動レベルおよび義歯の使用など）と独立して有意な差が認められた。クラスターBもハザード比2.7（95%信頼区間0.6～12.6）で，統計的な有意差は認められなかったが，クラスターAに比べて肺炎の発症率は高い傾向にあった。

さらに，発熱日数についてのオッズ比を多重ロジスティック回帰分析により比較したところ，クラスターAに比べてクラスターBで10.5（95%信頼区間2.0～55.5），クラスターCで4.4（95%信頼区間1.1～17.8），クラスターDで11.6（95%信頼区間2.3～57.8）と3つのクラスターすべてで有意に高い結果となった。

本研究では肺炎の発症はあくまで臨床医の診断に基づいたものであるが，わが国の公的医療保険制度では肺炎の臨床診断にX線写真の撮影は必ずしも必要ではないため，この研究の肺炎の定義は必ずしもX線写真によって裏づけられたものではない。したがって，肺炎の定義に若干の正確性を欠くことも考えられるが，肺炎と診断された被験者が何らかの健康障害を起こしていることに変わりはなく，広く肺炎類似の健康障害として捉えれば，的を外れた結果とは言えない。また，すべての患者の肺炎診断にX線写真の撮影を揃えた疫学

TRFs (Terminal restriction fragments)