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Oral Dysbiosis and Resulting Bacteremia Are Associated with Aortic Valve Diseases

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Abstract: Involvement of oral bacteria in the pathogenesis of distant organs has been shown. However, only a few reports have directly proven the involvement of oral bacteria in distant organ diseases. We attempted to analyze the hierarchy of bacterial species in the resected aortic valve by 16S metagenomic analysis and directly comparing their gene sequences with those in the oral cavity. Thirty-two patients with aortic stenosis or aortic regurgitation who underwent aortic valve replacement were enrolled in this study. Antibody titer against periodontal pathogenic bacteria in the patient's serum was analyzed. The genetic background and hierarchy of bacterial species on the subgingival plaque, the tongue dorsal surface, and the resected aortic valve was analyzed. Most patients with aortic valve disease have severe periodontal disease and show oral dysbiosis. Patients with aortic valve disease were shown to have more severe periodontal disease by the detection of antibodies against Socranscky's red-complex bacteria of periodontitis. Bacterial sequences of the aortic valve were sometimes identical to those of the oral cavity. The findings indicate that bacteria detected in the aortic valve may be infected through oral dysbiosis. Oral dysbiosis and the resulting bacteremia may be associated with the onset or progression of aortic valve disease.

Keywords: oral dysbiosis; aortic valve disease; infectious endocarditis; 16S metagenome analysis

1. Introduction

The involvement of oral bacteria with pathogenesis of distant organs have been shown [1,2]. Dysbiosis is known as pathological changes (type and/or proportion of bacteria) of commensal bacteria in some organs, which causes various diseases [3]. Recently, several reports have shown the association of oral bacteria or oral dysbiosis with diseases of other organs by the metagenome analysis and the molecular biological analysis [4,5].

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One known disease closely associated with oral bacteria is infectious endocarditis (IE), which causes verrucae on the endocardium. Aortic valve disease (AV) is a known cause of IE. Among aortic valve diseases (aortic stenosis (AS) and aortic regurgitation (AR)), AS is a condition in which blood flow from the left ventricle to the ascending aorta during systole is obstructed due to narrowing of the aortic valve orifice [6]. The causes include idiopathic degenerative sclerosis with calcification (congenital bicuspid valves prone to sclerosis) and rheumatic fever. Valve replacement or valvuloplasty is indicated when stenosis and regurgitation after AS/AR are severe [7].

We have previously reported that more than 60% of bacteria identified in the blood cultures of patients with IE are recognized as oral bacteria [8]. In our previous study, bacterial cultures of blood from patients with IE were compared with dental plaque cultures in the oral cavity to verify the identity of the oral bacteria and the causative bacteria of IE. The culture test was able to detect identical bacterial species; however, only in one case, the bacteria detected from dental plaque and blood had a complete match in the 16s rRNA gene sequence [8]. Although this study showed indirect evidence for so-called focal dental infections in distant organs, it was difficult to identify the causative bacteria by comparing the limited number of clones detected in the bacterial cultures.

In this study, we attempted to detect bacteria in the resected aortic valve, and analyze the hierarchy of bacterial species in the resected aortic valve by metagenomic analysis. Simultaneously, we analyzed the hierarchy of bacterial species in subgingival dental plaque, which causes periodontal disease, and in swabs of the tongue dorsal surface, which reflects the flora of the oral cavity in the same patients. Then we verified whether oral bacteria reached the aortic valve by directly comparing their gene sequences.

2. Results

2.1. Periodontitis and Aortic Valve Condition in Patients with Aortic Valve Disease

Thirty-two patients with AS or AR who underwent aortic valve replacement between May 2020 and March 2021 at Dokkyo Medical University Hospital were enrolled in this study. The patients' demographic data are presented in Table 1. These patients underwent oral examination and management before surgery, and periodontal disease was staged based on an intraoral examination, panoramic radiograph, and periodontal pocket depth [9].

Of the 32 patients, 15 were males, and 17 were females, with an average age of 74.15 years, 28 had severe AS, 2 had moderate to severe AS, and 2 had severe AS. Five patients had periodontal disease stage I, 2 patients had stage II, 5 had stage III, 10 patients had stage IV, and 10 patients were edentulous (Table 1). Twenty-six patients had calcification in the aortic valves and seven were suspected to have bicuspid valves based on transthoracic echocardiography. AVAi averaged 0.45±0.0012, SV averaged 78.93±78.60, and SVi averaged 49.23±27.57 (Table 1).

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Table 1. Demographic data of the AS/AR patient.

				Transthoratic echocardiography findings						Vulvelar findings				
Age	Gender	Stage of Periodontitis	Number of remaining tooth	Diagnosis	AVAi (cm^2/m^2)	LVEF	peakPG	SV(ml)	Svi(ml/m^2)	AR	MR	TR	PR	Structure
87	Female	IV	5	severeAS moderatePH	0.34	82	64	56	41	+	+	+	±	Cacification
77	Female	III	23	severeAS	0.62	74	95	90	58.4	-	-	±	-	Cacification
84	Female	NA	NA	moderateAS	0.64	64	22	39	25.9	±	++	++	±	Cacification
70	Female	IV	5	severeAS	0.26	65	88	49	29.7	±	±	−	−	Cacification Bicuspid valve
66	Male	IV	11	OMI moderateAS	0.41	40	42	39	26.5	−	+	+	+	Cacification
54	Female	III	21	severeAS	0.34	72	93	61	37.4	±	−	−	−	Cacification
66	Male	II	25	severeAS	ND	37	ND	141	94.1	+++	+	−	±	
65	Female	I	26	severeAS	0.4	44	54.17	47	32.6	+	±	+	±	Cacification
79	Female	NA	NA	severeAS	ND	45.2	56.3	ND	ND	+	+	±	−	Cacification
78	Female	NA	NA	severeAS	0.27	70	127.7	47	36.2	±	±	+	±	Cacification
58	Male	I	26	severeAS	0.36	34	94	78	44.5	+	±	±	++	Cacification Bicuspid valve s/o
74	Female	NA	NA	Post PMI severeAS	0.32	63	116	52	36.6	+	+	±	±	Cacification
78	Female	NA	0	severeAS	0.55	69	53	55	44.6	+	+~++	+	+	Cacification
72	Female	NA	0	severeAS	0.4	59	71	51	33.1	+	++	+	±	Cacification
76	Male	II	24	moderate - severeAS mildPH	0.42	84	37	35	24	+	++	+	±	Cacification
79	Male	IV	12	severeAS	0.44	66	104	96	56	+~++	±	+	+	No abnormal findings
83	Male	III	23	severeAS	0.42	66	77	65	42.4	±	±	+	±	Cacification Bicuspid valve
85	Male	NA	0	moderateAS	0.86	59	57	112	69.1	++~+++	+	+	±	Cacification
79	Female	NA	NA	moderate-severeAR	ND	61	ND	106	82.6	++~+++	±	++	+	No abnormal findings
77	Male	IV	5	severeAS	0.48	34	73	93	53	+	+~++	±	+	Cacification
74	Male	IV	16	moderate-severeAR	ND	74	16.5	107	55.4	++~+++	-	-	+	Cacification
86	Female	IV	11	moderate-severeAS	0.58	67	56	57	42.8	+	+	+	±	Cacification
75	Male	I	23	severeAR	ND	60	ND	195	120	+++	+	±	+	Cacification
71	Male	NA	0	severeAS	0.4	24	79	73	39.9	++	+	+	-	Cacification Bicuspid valv
78	Female	IV	8	severeAS	0.53	67	54	91	53.2	+	+	+	±	Cacification
64	Male	III	22	severeAS	0.56	56	85	116	60.6	++	±	-	+	Bicuspid valve
76	Male	NA	0	severeAS	0.53	70	71	105	53	±	±	−	-	Cacification
84	Female	I	28	severeAS mildPH	0.38	65	102	79	59	±	+	±	±	Cacification
72	Male	I	27	PostTAVI	ND	36	32	ND	ND	++	+	+	+	No abnormal findings
79	Female	IV	10	severeAS	0.28	62	129	56	38.4	−	+	+	−	No abnormal findings
65	Male	IV	11	severeAS	0.53	71	74	106	47	+	±	−	±	Cacification Bicuspid valve
61	Female	III	25	severeAS	0.38	75	83	71	40	-	-	±	-	Cacification Bicuspid valve

AS: aortic stenosis. AR: aortic regurgitation. AVA: aortic valve area. AVAi: AVA index. LVEF: left ventricular ejection fraction. peakPG: peak pressure gradient. SV: Stroke Volume. SVi: Stroke Volume index. MR: mitral regurgitation. TR: tricuspid regurgitation. PR: pulmonary regurgitation. PH: Pulmonary Hypertension. OMI: Old Myocardial Infarction. PMI: Pacemaker Implantation. TAVI: Transcatheter Aortic Valve Implantation. NA; not applicable. ND; not determined. In valvular findings, -; no sign of regurgitation, ±; trace level of regurgitation, +; mild regurgitation, ++; moderate regurgitation, +++; severe regurgitation

We compared the severity of periodontitis in the patients in this study and of the same age group from the results of the Survey of Actual Conditions of Dental Diseases in Japan [10]; 36.5% of the patients were classified as having mild disease in stages I and II, while 25.9% of patients with aortic disease were in the same category. In contrast, the percentage of patients with advanced periodontitis in stages III and IV and the edentulous jaw group was 74.1% in patients with aortic disease and 63.5% in the Survey of Dental Diseases in Japan (Figure 1). Although statistical comparisons were difficult because of the large difference in population size (32 patients in this study and 2378 patients in the

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survey), there was a tendency for more patients with aortic valve disease to have stage III or IV advanced periodontitis and edentulous jaws (odds ratio 1.602 [95%CI: 0.709 – 3.805]).

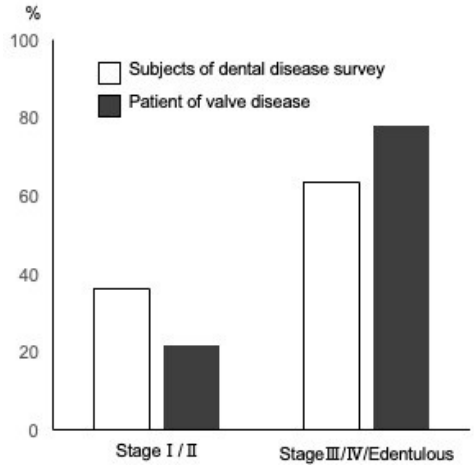

Figure 1. Periodontitis/edentulous status of patients with aortic valve disease. Stage represents the severity of periodontitis. Stage I and II were considered as early/mild periodontitis, while stage III and IVwere considered as advanced and moderate to severe periodontitis. The edentulous patient was considered as result of periodontitis progression.

2.2. Measurement of Serum Antibody Titer Against Periodontal Pathogenic Bacteria in Patients with Aortic Valve Disease

Periodontal pathogenic bacteria are classified into several categories (color-coded as red, orange, yellow, green, blue, and purple complexes) based on their importance in clinical pathogenesis by Socransky et al. [13], and bacteria classified in the red complex (*Porphyromonas gingivalis*, *Tannerella forsythia*, *Treponema denticola*) are considered to be important for the pathogenesis of periodontitis (Figure 2A). The radar chart in Figure 2B shows the mean antibody titers of various periodontal pathogenic bacteria in the serum of patients with aortic valve disease. Antibody titers against bacteria in the red complex category were significantly elevated in patients compared to healthy controls. A comparison of serum antibody titers against red complex bacteria by periodontitis stage in patients with aortic valve disease showed that antibody titers tended to increase with the progression of the periodontitis stage, and antibody titers to red-complex bacteria were positively correlated with the periodontitis stage (Figure 2C). A weak but positive correlation was also observed between the antibody titer against the bacteria of the orange complex and periodontitis stage (Figure 2C). Antibody titers against the bacteria of the blue and green complexes were weakly correlated with the periodontitis stage, although there was a tendency for antibody titers to increase.

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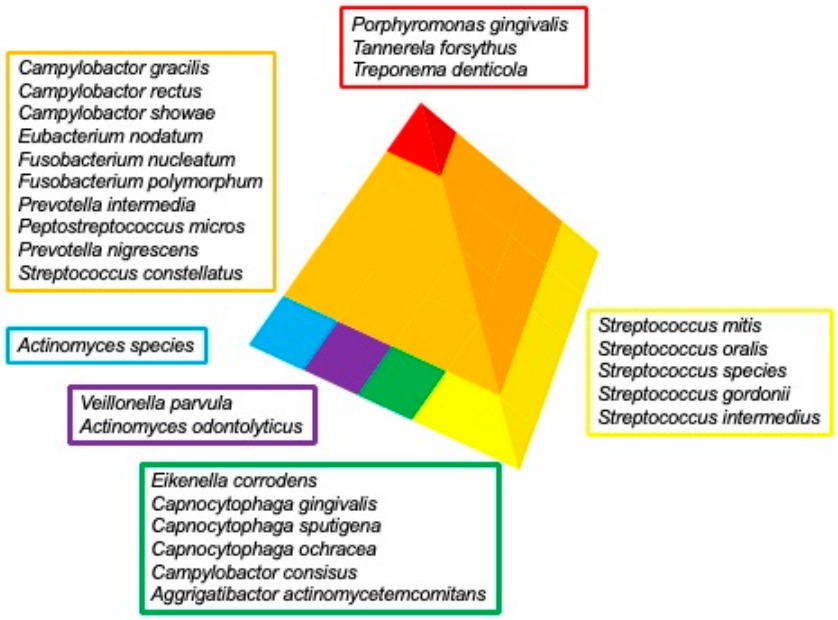
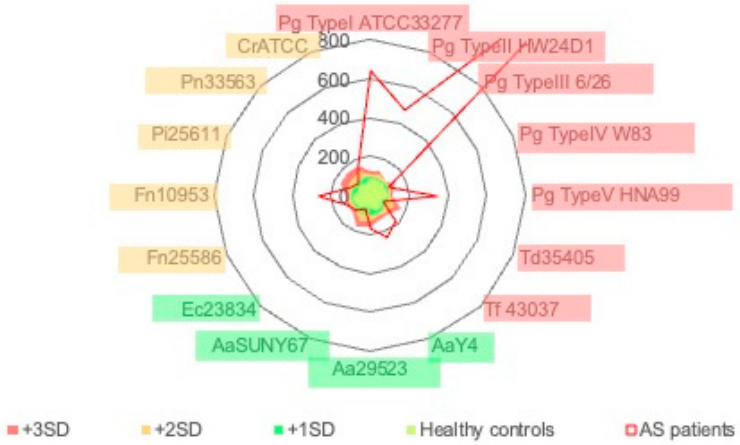
A



B



C

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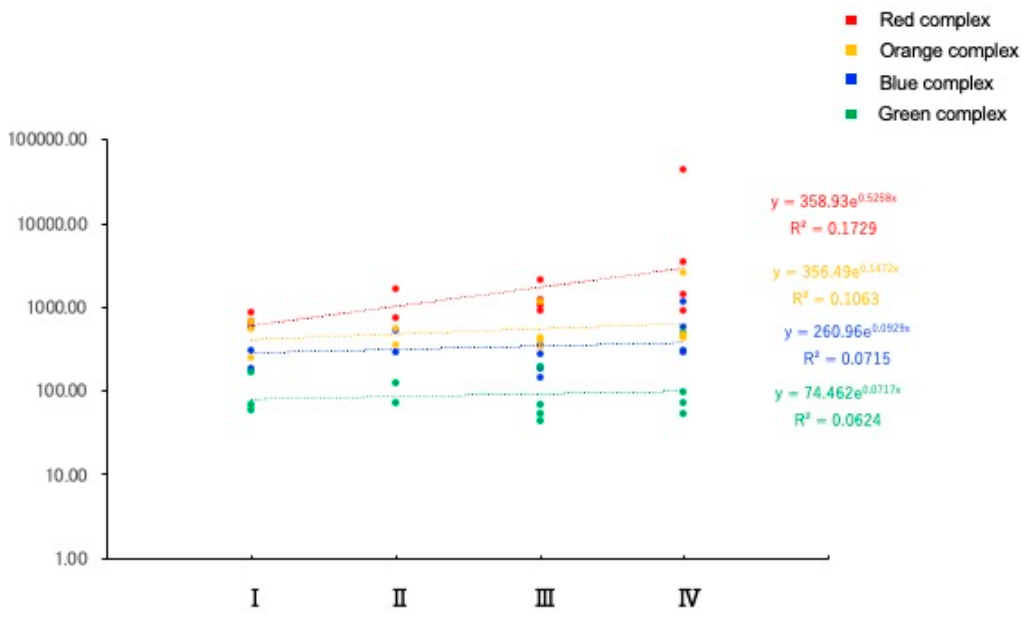

Figure 2. Status of antibody titer to periodontal pathogenic bacteria. (A)Schematic illustration of Periodontal pathogenic bacteria classified by Socransky's criteria. Periodontal pathogenic bacteria exist in defined order. They are divided in 6 categories, and each are coded by color. Blue, purple, green, and yellow complexes, forms the basic attachment to the body surfaces such as teeth and mucosa. Red complex are definite pathogens in periodontitis, while orange complex mainly functions as a connecting anchor for red complex members to the biofilm. (B) Average antibody titer against representative periodontal pathogenic bacteria. Antibody titer against each pathogenic bacteria are standardized to average value of healthy controls. Red area in radar chart demonstrates +3 S.D. titer from healthy controls, orange area, +2 S.D., green area; +1 S.D, respectively. Average of AS/AR patients in this study are shown in red line. (C) Correlation of antibody titers against the periodontal pathogenic bacteria and periodontal stage of AS/AR patient. Sum of each red, orange, blue, and green complexes of each patients tested are plotted.

2.3. Taxonomy Analysis

Bacterial 16S rRNA was detected in 12 aortic valves of 32 patients examined using PCR. We obtained sufficient DNA for amplicon sequencing analysis in six cases. Taxonomic analysis was performed to identify ASVs with > 70% homology. The flora composition based on the read count of each ASV is shown in (Fig. S1). Surprisingly, several bacterial species were detected in the resected aortic valve. As expected, the flora of the tongue and periodontal pocket dental plaque were similar in many cases; however, the flora detected in the aortic valve showed a different pattern.

2.4. α-Diversity and β-Diversity

Examination of α-diversity and β-diversity indicated that the bacterial flora in the resected aortic valve tended to be similar, although not identical, to the composition of the oral flora. We examined the α-diversity of the plaques, tongue swabs, and excised aortic valves (Fig. S2). There was no significant difference in the diversity within each flora detected at each site ($p = 0.547$). This suggests that a wide variety of bacteria are present in the resected aortic valve, and that, like the oral environment, the flora may be diverse. When we examined the β-diversity among the flora (Fig. S3), we found that the vectors of diversity of the flora in the three groups were different ($p = 0{\cdot}001$): plaque, tongue, and resected aortic valve. Among the resected aortic valves, we recognized two groups with different vectors of diversity, and among the two groups, three cases, AS 14, 20, and 25, were considered to have flora similar to those of the oral-derived specimens, being closer to the oral bacteria. The frequency of detection of ASV in AS 18, 28, and 30 was higher than that in the Unassigned group, which may have been a factor in the division into two groups. (Although the Gene

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analyzer detected the expected size of PCR products in these three samples at the 1stPCR stage, it was insufficient in quantity. As the primary purpose of this study was to examine whether bacteria were present in the resected aortic valve, we performed 1st PCR again using the 1st PCR product as a PCR template. This increased nonspecific amplification, and unassigned ASVs became more frequent. However, from the viewpoint of Axis 2 and 3, there was a similar trend to that of the oral-derived specimens.

2.5. Characteristics of Bacteria Detected in the Tongue, Dental Plaque, and Resected Aortic Valve

The 195 bacteria detected in the tongue, dental plaque, and resected aortic valve specimens were ranked according to read count. The lists of the top 25 bacteria are shown in Figure 3A, B, and C. Among the bacteria listed for resected aortic valves, 48% (16-64%) of the 35 species were associated with the oral cavity, and 18% (4-28%) were periodontal pathogenic bacteria (color-coded as red, orange, yellow, green, blue, and purple) according to Socransky's classification [14]. In cases 20, 25, and 30, a significant number of red and orange bacteria were included in the flora considered endemic to the oral cavity detected on the dorsal surface of the tongue. In cases 20 and 25, red and orange bacteria were detected in the aortic valve. In Case 30, neither red nor orange complex bacteria were detected in the aortic valve. In cases 14, 18, and 28, the bacterial flora detected on the dorsal surface of the tongue contained no red and only a few orange bacteria. In contrast, red and orange bacteria were detected in the aortic valve.

A

list of bacterias related to periodontitis	Ranking	AS-14V		AS-18V		AS-20V		AS-25V		AS-28V		AS-30V	
red Porphyromonas gingivalis	1	21743	Unassigned	9652	g__Staphylococcus	8038	g__Staphylococcus	7624	Capnocytophaga_sp.	17720	g__Staphylococcus	21298	Unassigned
Treponema denticola	2	7035	d__Bacteria	7813	g__Anaerococcus	5072	g__Streptococcus	7018	g__Cutibacterium	17324	Unassigned	9395	d__Bacteria
Tannerella forsythensis	3	2747	g__Parvimonas	6836	g__Finegoldia	4393	Prevotella_oulorum	6683	Dolosigranulum_pigrum	11532	d__Bacteria	2757	g__Staphylococcus
Orange Campylobacter gracillis	4	1398	Prevotella_sp.	6272	g__Paracoccus	4385	g__Cutibacterium	6570	g__Alloprevotella	747	g__Anaerobacillus	966	Parabacteroides_goldsteinii
Campylobactor rectus	5	1175	Solobacterium_moorei	4172	g__Chloroplast	4312	g__Centipeda	4680	g__Enhydrobacter	587	Fusobacterium_periodontium	604	g__Lawsonella
Campylobactor showae	6	986	g__Acinetobacter	3231	Prevotella_oulorum	3801	g__Alloprevotella	4295	g__Lawsonella	549	Lactobacillus_iners	502	Kocuria_marina
Eubacterium nodatum	7	807	Porphyromonas_endodontalis	3140	Prevotella_denticola	3623	g__Porphyromonas	3960	Veillonella_sp.	433	g__Cutibacterium	486	Cryptomeria_japonica
Fusobacterium nucleatum	8	724	Phascolarctobacterium_succinatutens	2467	g__Enhydrobacter	3042	g__Lawsonella	3895	Prevotella_loescheii	353	g__Rothia	450	Cyanothece_sp.
Fusobacterium polymorphum	9	586	g__Enhydrobacter	2332	g__Streptococcus	2835	Porphyromonas_pasteri	3867	Acinetobacter_baumannii	280	Bacillus_pseudofirmus	410	g__Faecalibaculum
Prevotella intermedia	10	525	g__Peptoniphilus	2276	Capnocytophaga_ochracea	2584	Haemophilus_parainfluenzae	3148	Prevotella_nigrescens	258	g__Fusobacterium	377	Sphingomonas_sp.
Peptostreptococcus micros	11	497	f__Veillonellaceae	1606	g__Veillonella	2529	Capnocytophaga_sputigena	2875	Prevotella_denticola	256	d__Eukaryota	353	g__Nocardioides
Prevotella nigrescens	12	459	g__Pseudoramibacter	1529	Capnocytophaga_gingivalis	2053	f__Paracoccus	2616	g__Clostridia_UCG-014	243	g__Streptobacillus	312	g__Anaerobacillus
Streptococcus constellatus	13	458	g__Peptostreptococcus	1518	Prevotella_nigrescens	1862	Prevotella_denticola	2334	g__Neisseria	191	g__Alloprevotella	303	d__Eukaryota
Yellow Streptococcus gordonii	14	450	g__Atopobium	1507	Corynebacterium_tuberculostearicum	1758	g__Amarricoccus	2148	g__Abiotrophia	164	g__Lawsonella	275	g__Streptococcus
Streptococcus intermedius	15	441	Bacteroidales_oral	1037	g__Paenibacillus	1660	Candidatus_Saccharibacteria	1828	g__Streptococcus	164	g__Cellulomonas	236	g__Cellulomonas
Streptococcus mitis	16	441	Akkermansia_muciniphila	987	Treponema_medium	1613	g__Fusobacterium	1633	g__F0058	162	g__Burkholderia-Caballeronia-Paraburkholderia	216	uncultured_Enterobacteriaceae
Streptococcus oralis	17	431	g__Paracoccus	832	g__Prevotella	1599	g__Pedobacter	1448	Capnocytophaga_ochracea	121	f__Micrococcaceae	204	Cicer_arietinum
Streptococcus sanguis	18	311	g__Staphylococcus	830	g__Peptoniphilus	1570	g__Skermanella	1182	f__Pasteurellaceae	103	Fusobacterium_sp.	194	g__Mitochondria
Streptococcus sp.	19	307	Dialister_invisus	801	g__Rothia	1489	g__Porphyromonas	1121	g__Paracoccus	90	g__Neisseria	188	g__Lactobacillus
Green Aggregatibacter actinomycetescomitans	20	294	g__Anaerobacillus	786	g__Cutibacterium	1397	g__Leptotrichia	1053	g__Pseudomonas	58	g__Porphyromonas	186	uncultured_Spartobacteria
Capnocytophaga gingivalis	21	274	g__Pseudomonas	772	g__Dermabacter	1389	Veillonella_sp.	905	Kocuria_marina	49	g__Escherichia-Shigella	175	g__Micrococcus
Capnocytophaga sputigena	22	270	Porphyromonas_sp.	680	Prevotella_shahii	1381	Leptotrichia_buccalis	697	g__Actinomyces	41	g__Aerosphaera	175	g__Anaerococcus
Capnocytophaga ochracea	23	244	Filifactor_alocis	612	g__Fusobacterium	1304	g__Micrococcus	687	g__Streptobacillus	37	Murdochiella_asaccharolytica	171	g__Ezakiella
Campylobacter concisus	24	240	g__Kocuria	600	g__Othonobacter	1161	g__Spirosoma	574	g__Solobacterium	32	g__Streptococcus	170	g__Methylobacterium-Methylorubrum
Eikenella corrodens	25	236	g__Anaerococcus	566	Prevotella_loescheii	1042	g__Alloprevotella	60	Corynebacterium_tuberculostearicum	31	g__Bradyrhizobium	158	Corynebacterium_tuberculostearicum

B

list of bacterias related to periodontitis	Ranking	AS-14P	属種名	AS-18P		AS-20P		AS-25P		AS-28P		AS-30P	
red Porphyromonas gingivalis	1	11798	g__Veillonella	19179	Selenomonas_noxia	5688	g__Saccharimonadaceae	9415	g__Neisseria	28191	Streptococcus_salivarius	9859	g__Neisseria
Treponema denticola	2	5142	Prevotella_melaninogenica	6207	g__Saccharimonadaceae	5274	g__F0058	8062	g__Veillonella	14266	g__Streptococcus	4906	Fusobacterium_periodontium
Tannerella forsythensis	3	4948	g__Haemophilus	4906	Prevotella_shahii	4962	g__Fusobacterium	4745	Prevotella_melaninogenica	11266	Veillonella_atypica	4438	Prevotella_loescheii
Orange Campylobacter gracillis	4	4441	g__Streptococcus	4340	Leptotrichia_hofstadii	4288	Anaeroglobus_geminatus	4153	g__Fusobacterium	7423	Lactobacillus_fermentum	4225	g__Veillonella
Campylobactor rectus	5	4345	Prevotella_nigrescens	3909	Corynebacterium_matruchotii	2607	Candidatus_Saccharibacteria	3093	Streptococcus_sanguinis	3666	Prevotella_histicola	4170	g__Lautropia
Campylobactor showae	6	4042	Haemophilus_parainfluenzae	3777	Selenomonas_artemidis	2447	Leptotrichia_buccalis	2940	Porphyromonas_gingivalis	2925	g__Rothia	3702	Capnocytophaga_gingivalis
Eubacterium nodatum	7	3419	uncultured_Prevotella	3234	Capnocytophaga_granulosa	2336	g__Centipeda	2929	Neisseria_elongata	1998	g__Granulicatella	3452	g__Fusobacterium
Fusobacterium nucleatum	8	3049	Fusobacterium_nucleatum	3001	Campylobacter_gracilis	2201	g__Leptotrichia	2901	g__Haemophilus	1349	Rothia_mucilaginosa	3404	g__Lachnoanaerobaculum
Fusobacterium polymorphum	9	2588	g__Rothia	2275	g__Fusobacterium	2100	Leptotrichia_wadei	2498	Prevotella_loescheii	1263	g__Chloroplast	2793	g__Haemophilus
Prevotella intermedia	10	2141	g__Actinomyces	1956	g__TM7x	1918	Campylobacter_gracilis	2269	Neisseria_oralis	688	g__Veillonella	2764	Neisseria_elongata
Peptostreptococcus micros	11	1704	Prevotella_histicola	1561	g__Streptococcus	1859	Prevotella_genomosp.	2047	Prevotella_nigrescens	488	Alloscardovia_omnicolens	1980	g__Selenomonas
Prevotella nigrescens	12	1700	Dialister_invisus	1524	Neisseria_bacilliformis	1797	Prevotella_nigrescens	1428	Prevotella_oris	464	g__Lactobacillus	1915	Streptococcus_sanguinis
Streptococcus constellatus	13	1691	Prevotella_oris	1481	Capnocytophaga_ochracea	1582	Treponema_socranskii	1353	Prevotella_denticola	412	g__Mitochondria	1866	Capnocytophaga_ochracea
Yellow Streptococcus gordonii	14	1199	Prevotella_pallens	1392	Megasphaera_micronuciformis	1567	g__Tannerella	1287	Prevotella_intermedia	373	Prevotella_salivae	1560	Corynebacterium_matruchotii
Streptococcus intermedius	15	1195	Prevotella_genomosp.	1278	g__Veillonella	1486	Alloprevotella_tannerae	1152	Candidatus_Saccharibacteria	287	g__Gemella	1458	g__Johnsonella
Streptococcus mitis	16	1128	Veillonella_parvula	1231	g__Selenomonas	1454	Prevotella_oralis	1140	Anaeroglobus_geminatus	279	Schaalia_odontolytica	1260	g__Selenomonadaceae
Streptococcus oralis	17	1107	Megasphaera_micronuciformis	1173	g__Capnocytophaga	1253	g__Prevotella	1037	Capnocytophaga_sp.	235	Campylobacter_concisus	1222	g__Campylobacter
Streptococcus sanguis	18	1079	g__Atopobium	1172	Tannerella_sp.	1259	g__TM7x	1037	g__Clostridia_UCG-014	166	Oryza_sativa	1221	Prevotella_melaninogenica
Streptococcus sp.	19	1070	Dialister_pneumosintes	1099	Selenomonas_sp.	1161	g__Leptotrichia	987	Alloprevotella_tannerae	146	g__Atopobium	1193	Capnocytophaga_sputigena
Green Aggaregatibacter actinomycetescomitans	20	1040	Solobacterium_moorei	1097	g__Lachnoanaerobaculum	1052	g__Porphyromonas	832	f__Neisseriaceae	107	g__Actinomyces	1189	Capnocytophaga_leadbetteri
Capnocytophaga gingivalis	21	1037	g__Alloprevotella	1082	g__Cardiobacterium	1050	Prevotella_marshii	795	Capnocytophaga_leadbetteri	99	g__Klebsiella	1185	Porphyromonas_pasteri
Capnocytophaga sputigena	22	890	Capnocytophaga_gingivalis	1038	Neisseria_elongata	997	Capnocytophaga_leadbetteri	791	g__Lautropia	96	Lactobacillus_gasseri	1175	g__Abiotrophia
Capnocytophaga ochracea	23	864	g__Neisseria	901	g__Kingella	865	Corynebacterium_matruchotii	750	Prevotella_genomosp.	83	g__Stomatobaculum	1098	g__Leptotrichia
Campylobactor concisus	24	834	g__Fusobacterium	821	Prevotella_genomosp.	822	f__Selenomonadaceae	714	Selenomonas_sputigena	83	g__Acinetobacter	1061	f__Lachnospiraceae
Eikenella corrodens	25	829	uncultured_Prevotella	625	Capnocytophaga_leadbetteri	728	Schwartzia_sp.	700	f__Lachnospiraceae;	80	Pyropia_yezoensis	1039	g__Johnsonella

C

list of bacterias related to periodontitis	Ranking	AS-14T		AS-18T		AS-20T		AS-25T		AS-28T		AS-30T	
red Porphyromonas gingivalis	1	11585	Prevotella_melaninogenica	35215	g__Neisseria	13883	g__Rothia	9703	g__Neisseria	13351	g__Streptococcus	13773	g__Neisseria
Treponema denticola	2	8842	g__Veillonella	6806	g__Veillonella	5298	g__Streptococcus	8093	g__Veillonella	10942	Streptococcus_salivarius	3550	g__Lachnoanaerobaculum
Tannerella forsythensis	3	8581	Fusobacterium_periodontium	3194	g__Haemophilus	3976	g__Campylobacter	7379	Haemophilus_parainfluenzae	9978	Veillonella_atypica	3510	Porphyromonas_pasteri
Orange Campylobacter gracillis	4	7567	g__Neisseria	3151	Prevotella_melaninogenica	3469	Porphyromonas_gingivalis	4931	Prevotella_pallens	8167	Rothia_mucilaginosa	3269	g__Rothia
Campylobactor rectus	5	5733	g__Lachnoanaerobaculum	3086	Fusobacterium_periodontium	2278	g__Actinomyces	4911	g__Streptococcus	7005	Prevotella_histicola	3119	Fusobacterium_periodontium
Campylobactor showae	6	3281	g__Haemophilus	2708	g__Streptococcus	2227	Lactobacillus_reuteri	2459	g__Fusobacterium	6617	g__Klebsiella	3111	g__Streptococcus
Eubacterium nodatum	7	3262	Veillonella_atypica	2405	Prevotella_pallens	2163	Tannerella_forsythia	2446	Fusobacterium_periodontium	4582	g__Oribacterium	3098	Haemophilus_parainfluenzae
Fusobacterium nucleatum	8	3228	Prevotella_pallens	2139	g__Leptotrichia	2023	Streptococcus_salivarius	2329	Prevotella_melaninogenica	3304	Megasphaera_micronuciformis	2869	Neisseria_elongata
Fusobacterium polymorphum	9	2640	Campylobacter_concisus	1888	Prevotella_shahii	1993	g__Chloroplast	2292	g__Leptotrichia	1603	g__Veillonella	2754	g__Alloprevotella
Prevotella intermedia	10	2354	g__Streptococcus	1655	Rothia_mucilaginosa	1859	Porphyromonas_endodontalis	1886	Prevotella_jejuni	1497	Prevotella_salivae	2699	g__Porphyromonas
Peptostreptococcus micros	11	2295	Capnocytophaga_gingivalis	1489	g__Alloprevotella	1820	Lactobacillus_gasseri	1435	g__TM7x	1250	g__Stomatobaculum	2671	Lachnoanaerobaculum_umeaense
Prevotella nigrescens	12	2106	g__Oribacterium	1366	g__Rothia	1599	Veillonella_atypica	1302	Neisseria_elongata	1106	Lactobacillus_fermentum	2585	g__Veillonella
Streptococcus constellatus	13	1895	uncultured_Bacteroidetes	1180	g__Oribacterium	1566	g__Veillonella	1240	g__Haemophilus	1051	g__Rothia	2464	g__Granulicatella
Yellow Streptococcus gordonii	14	1175	Megasphaera_micronuciformis	1105	Leptotrichia_sp.	1470	Lactobacillus_fermentum	1207	Porphyromonas_gingivalis	749	Alloscardovia_omnicolens	1562	g__Solobacterium
Streptococcus intermedius	15	1034	Solobacterium_moorei	838	g__Solobacterium	1426	Lactobacillus_salivarius	1163	Solobacterium_moorei	703	Schaalia_odontolytica	1442	g__Haemophilus
Streptococcus mitis	16	956	Eubacterium_sulci	773	Veillonella_atypica	1423	g__Granulicatella	1150	Porphyromonas_pasteri	682	g__Actinomyces	1335	g__Stomatobaculum
Streptococcus oralis	17	856	g__Leptotrichia	715	g__Lachnoanaerobaculum	1206	g__F0058	1125	g__Leptotrichia	635	g__Granulicatella	1297	Schaalia_odontolytica
Streptococcus sanguis	18	782	Prevotella_histicola	631	Campylobacter_concisus	1133	Treponema_lecithinolyticum	1116	g__Porphyromonas	567	Campylobacter_concisus	1087	g__Clostridia_UCG-014
Streptococcus sp.	19	772	Schaalia_odontolytica	597	Capnocytophaga_gingivalis	1116	Anaeroglobus_geminatus	1042	g__Alloprevotella	431	Solobacterium_moorei	1011	g__Porphyromonas
Green Aggaregatibacter actinomycetescomitans	20	711	g__Catonella	538	g__Bergeyella	1089	Streptococcus_anginosus	1025	g__Aggregatibacter	351	g__Atopobium	929	g__Oribacterium
Capnocytophaga gingivalis	21	667	Prevotella_scopos	523	Schaalia_odontolytica	1033	g__Fusobacterium	957	Megasphaera_micronuciformis	246	g__Lachnoanaerobaculum	763	g__Moraxella
Capnocytophaga sputigena	22	663	g__Atopobium	490	Porphyromonas_pasteri	1003	Corynebacterium_matruchotii	916	Eubacterium_sulci	209	f__Enterobacteriaceae	764	Veillonella_rogosae
Capnocytophaga ochracea	23	646	Haemophilus_parainfluenzae	457	Streptococcus_salivarius	959	Schaalia_odontolytica	864	g__Campylobacter	196	g__Lactobacillus	742	Prevotella_aurantiaca
Campylobactor concisus	24	607	Prevotella_nanceiensis	420	Selenomonas_sp.	927	Candidatus_Saccharibacteria	828	Prevotella_intermedia	139	g__Gemella	662	g__Johnsonella
Eikenella corrodens	25	590	g__Peptococcus	411	g__Fusobacterium	809	Prevotella_denticola	819	Prevotella_salivae	81	Actinomyces_graevenitzii	623	g__Lautropia

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Figure 3. Top 25 bacteria listed by read count in each sample. Bacteria related to oral cavity are highlighted. The background color represents the Socransky's periodontal pathogens. (A) List of aortic valve samples. (B) List of dental plaque samples. (C) List of tongue samples.

2.6. Identification of the Same Bacterial Clones in the Tongue, Dental Plaque, and Resected Aortic Valve

The same ASV was detected in the tongue, dental plaque, and the resected aortic valve (Figure 4). This indicates that bacteria with the same genetic background, that is, the same clones of the bacteria, are present in the oral cavity and aortic valve. A total of 2,524 independent ASVs were detected in all samples. Forty bacteria were found to have identical ASVs in the excised aortic valve and oral cavity (dorsal surface of the tongue and/or dental plaque). Fourteen bacteria were detected with identical ASVs from the three sites of the tongue, dental plaque, and excised aortic valve, and 26 bacteria were detected with identical ASVs from the excised aortic valve and tongue or dental plaque (Figure 4). Twelve of the 40 bacteria were periodontal pathogenic bacteria, according to Socransky's classification.

		#14			#18			#20			#25			#28			#30		
Taxonomy	Confidence	P	T	V	P	T	V	P	T	V	P	T	V	P	T	V	P	T	V
Streptococcus_salivarius	0.802	53	378	133	0	450	0	0	2021	0	0	6	0	28191	10942	0	0	0	0
g__Streptococcus	1.000	4262	1632	0	777	847	1774	503	3898	1559	258	3745	1734	6153	824	32	676	849	275
g__Rothia	1.000	2588	0	0	0	0	660	0	15250	495	127	23	0	0	0	353	13	0	49
g__Staphylococcus	1.000	0	0	311	0	0	4430	0	0	0	0	3	0	46	45	8633	0	0	1556
Prevotella_nigrescens	1.000	1729	0	0	0	0	0	1797	39	0	2047	504	1888	0	0	0	351	47	0
Neisseria_elongata	0.998	0	0	0	0	0	0	0	0	0	1422	1051	0	0	8	0	2733	2869	59
Prevotella_oulorum	1.000	22	0	0	0	0	2122	0	119	4273	0	0	0	0	0	0	0	0	0
Clostridia_UCG-014	1.000	766	408	0	0	21	0	143	0	0	1037	150	2616	0	5	0	241	1087	0
uncultured_bacterium	0.951	0	0	0	63	4	0	2476	161	0	0	113	1633	0	0	0	264	4	0
uncultured_bacterium	0.789	0	0	0	57	0	0	120	0	4312	0	0	0	0	0	0	0	0	0
uncultured_bacterium	1.000	0	0	0	0	0	0	0	0	301	586	44	2148	0	0	0	1175	28	0
g__Veillonella	1.000	986	0	0	254	0	152	75	440	0	2184	125	0	0	0	0	0	0	0
Prevotella_denticola	0.998	0	0	0	58	0	1614	0	676	1763	0	0	0	0	0	0	0	0	0
uncultured_Bacteroidetes	1.000	76	1127	0	2	1489	125	22	19	0	25	1042	0	0	0	0	0	0	0
Veillonella_sp.	0.964	0	0	0	0	0	0	0	0	0	0	85	3836	0	0	0	0	0	0
Capnocytophaga_sputigena	0.998	102	6	0	0	0	0	71	0	2219	0	646	0	0	0	0	641	218	0
Leptotrichia_buccalis	0.955	0	0	0	0	0	0	2242	0	743	0	251	0	0	0	0	0	0	0
g__Fusobacterium	1.000	0	0	0	0	0	0	1313	0	1613	0	265	0	0	0	0	0	0	8
g__Parvimonas	1.000	25	0	2660	0	0	0	5	119	0	64	151	0	0	0	0	0	0	0
Prevotella_nigrescens	1.000	2616	26	0	49	0	152	0	43	0	0	0	0	0	0	0	0	0	0
Prevotella_nigrescens	1.000	0	0	0	160	15	1174	0	135	0	0	0	1260	0	0	0	0	0	0
Capnocytophaga_gingivalis	0.999	0	1374	0	154	13	1017	0	74	0	0	0	0	0	0	0	0	0	0
g__Peptostreptococcus	1.000	758	519	362	0	101	0	57	33	0	219	201	0	0	0	0	9	101	0
Corynebacterium_matruchotii	0.994	0	0	0	1254	40	199	0	663	0	0	0	0	0	0	0	0	0	0
g__Streptococcus	1.000	0	100	17	0	755	0	0	90	0	0	490	0	286	51	0	0	160	0
Capnocytophaga_sp.	0.907	0	0	0	0	0	0	0	0	0	514	0	1301	0	0	0	0	0	0
Corynebacterium_tuberculostearicum	0.879	0	0	0	0	0	1507	0	26	8	0	0	60	0	0	22	0	0	158
Streptococcus_gordonii	0.862	237	0	0	100	0	189	0	272	0	497	252	0	0	0	0	0	0	0
g__Veillonella	1.000	13	0	0	295	0	309	0	14	0	846	0	0	0	0	0	0	0	0
g__Pseudoramibacter	1.000	29	0	459	0	0	0	0	664	0	0	7	0	0	0	0	0	0	0
g__Actinomyces	1.000	330	0	0	0	0	0	0	0	0	194	0	482	0	0	0	31	0	0
g__Actinomyces	1.000	16	0	0	0	0	0	0	124	311	0	0	0	0	0	0	0	0	0
Solobacterium_moorei	0.807	413	0	1175	10	0	0	71	0	0	41	71	0	0	0	0	0	0	0
Dialister_invisus	0.873	1700	6	307	0	7	91	720	450	0	558	153	0	0	0	0	52	7	0
Filifactor_alocis	0.939	10	8	202	0	10	0	8	0	0	0	314	0	0	0	0	0	0	0
Dialister_pneumosintes	0.999	1070	0	40	0	0	0	28	130	0	203	72	0	0	0	0	4	0	0
Haemophilus_parainfluenzae	0.788	4042	646	0	0	0	3	14	0	2584	0	7379	0	0	16	0	659	2666	0
Capnocytophaga_sp.	0.924	0	0	0	197	0	0	0	0	0	523	0	5867	0	0	0	109	0	0
Fusobacterium_periodonticum	0.986	172	7651	0	29	1518	0	83	0	0	0	2446	0	0	12	587	4906	2802	0
g__Burkholderia-Caballeronia-Paraburkholderia	0.998	0	0	18	0	0	0	0	11	0	0	0	0	6	0	162	0	0	0

Figure 4. List of bacteria which the ASV was matched in dental plaque, tongue, and valve. Matched samples are highlighted by red background. The numbers in the tables represents the read count of each bacterium.

3. Discussion

In this study, we found the following points: [1] patients with aortic valve disease were more likely to have periodontitis and more of them had severe disease or edentulous jaws; [2] serum titers of antibodies against periodontal pathogenic bacteria and red complexes were high in patients with aortic valve disease; [3] Bacterial DNA was detectable in the resected aortic valve of 12/32 (37.5%) patients with aortic valve disease; [4] a wide variety of bacteria were detected in the resected aortic valves of 6 patients who underwent amplicon sequencing analysis; [5] 40 identical bacteria were found in both the resected aortic valve and oral bacterial ASVs; [6] among the 40 identical bacteria,12 were periodontal pathogenic bacteria according to Socransky's classification. These results strongly suggests that in patients with current periodontitis or previous periodontitis, oral bacteria invade the body and settle in the aortic valve via the blood stream.

In some cases (cases 20, 25, and 30), bacteria in the red and orange complexes were significant in the flora of the oral cavity. It was endemic to the oral cavity and was detected on the dorsal surface

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of the tongue, suggesting that dysbiosis had occurred. In cases 20 and 25, bacteria in the red and orange complexes were also detected in the aortic valve. In contrast, the dorsal surface of the tongue contained less orange complex bacteria in cases 14, 18, and 28, without red complexes. Nevertheless, red and orange complexes were detected in the aortic valve, suggesting that colonization of the aortic valve via bacteremia from periodontal bacteria in the periodontal pocket occurs even in the absence of oral bacterial dysbiosis. The identity of the bacteria detected in the oral cavity (dorsal surface of the tongue or periodontal pockets) and the aortic valve was examined, and in cases 14, 18, 20, and 25, a significant number of bacteria were detected from both sources. Only Staphylococcus and Neisseria were detected in cases 28 and 30, respectively, suggesting that oral bacteria may be less involved.

In many cases, Streptococcus, which appears to be an early colonizer, and subsequent dental plaque constituent bacteria were also present in the aortic valve, suggesting that they may be involved in bacterial colonization. However, some ASV species that were detected in the resected aortic valve were not detected in the oral cavity. Because oral dysbiosis has been present for a considerable period and the timing of bacterial invasion and settlement in the aortic valve does not always coincide because of repeated acute conversion and remission of periodontal disease, it is thought that there were bacterial clones that existed in the oral cavity but whose ASVs did not coincide.

Although there have been many reports on the detection of oral bacteria in distant organs using PCR [8,15–21], a few reports have verified whether the bacteria detected in the oral cavity and distant organs are the same clone [8]. For example, Zeibolz et al. examined the presence of pathogenic periodontal bacteria in aortic valves using PCR [15]. The relationship between oral bacteria and diseases of distant organs is also well known. Periodontal pathogens are known to be risk factors for atherosclerosis [16,17] and, as a result, are thought to be involved in the development of several cardiac diseases, including infective endocarditis [18]. Streptococcus mutans, a well-known caries-causing bacterium, is involved in the development of cerebral hemorrhage [19–21]. Although these findings are valuable as circumstantial evidence that oral bacteria can infect distant organs via the bloodstream, it is not easy to examine whether the diseases in oral cavity, such as periodontitis and dental caries can be the gateway to entry. In this study, we found that identical clones of bacteria were present in the oral cavity, distant organs, and the aortic valve. However, at present, we did not clarify whether oral bacteria caused aortic valve disease or colonized the injured aortic valve.

The importance of oral management during surgery and several medical treatments have recently been reported [22]. However, there is no clear direct evidence that bacteria in the oral cavity can induce secondary infections in other organs. Accumulated experience strongly suggests that oral lesions (dental caries reaching the root canal or periodontal disease) may serve as a gateway for bacterial invasion into the body, and studies providing direct evidence have increased. In this study, we detected bacteria with the same genetic background, that is, the same clone of bacteria, in the oral cavity and resected aortic valve of patients with aortic valve disease. Oral dysbiosis and the resulting bacteremia may be associated with the onset or progression of aortic valve disease.

4. Materials and Methods

4.1. Patients

Thirty-two patients with AS or AR who underwent aortic valve replacement between May 1, 2020 and March 31, 2021 at Dokkyo Medical University Hospital were enrolled in this study. This study was approved by the Ethics Committee of Dokkyo Medical University (approval no. R-37-20J). Patients were included in the study only when consent was obtained from the patient or the key person. In cases where the patients themselves could not make a decision; we explained the details of the research to the key participants in the study. In this study, we obtained genetic information from microorganisms, but not human-derived information.

4.2. Measurement of Serum Antibody Titer Against Periodontal Pathogenic Bacteria

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Serum antibody titers were measured according to a previously reported [9]. Serum from the preoperative blood collection of patients was isolated and stored at -80°C. Serum was collected from patients during surgery and stored at -80°C. Serum IgG antibody titers against periodontal pathogenic bacteria were determined by ELISA using strains of bacteria that had already been isolated and identified. The antibody titer against each bacterium was expressed as a relative value using the antibody titer in the sera of adults without advanced periodontal disease as the standard [9]. We examined the stages of periodontitis and antibody titers against periodontal pathogenic bacteria using the Kruskal–Wallis test. The correlation between the antibody titer and stage of periodontitis was analyzed.

4.3. DNA Extraction from the Aortic Valve or Oral Bacteria

Aortic valves were stored in 1.5 ml microtubes at -80°C immediately after surgical excision. Isospin Tissue DNA (Nippon Gene Inc., Tokyo, Japan) was used to extract DNA from excised aortic valves. Samples of the dorsal surface of the tongue, representing the flora of the oral cavity, were collected using cotton swabs. The swabs were rubbed on the dorsal surface of the tongue and stored in conical tubes at -80°C until DNA extraction. In addition, subgingival plaque samples were collected from sites with obvious inflammatory conditions, such as periodontitis, using a manual scaler and stored in 1.5-ml microtubes at -80°C until DNA extraction. DNA was extracted from plaque and tongue swab samples using the Isoil DNA extraction kit following the manufacturer's instructions (Nippon Gene Inc., Tokyo, Japan). The plaques were added directly to the extraction buffer, and the swab tips were cut into the extraction buffer, thoroughly agitated, and centrifuged to obtain the supernatant.

4.4. Confirmation of Bacterial DNA in the Extracted Samples

Extracted DNA was confirmed using primers against bacterial 16S rRNA. The primers used were 16S rRNA-27f: 5′-AGAGAGTTTGATCCTGGCTCAG-3′,16S rRNA-1492r: 5′-ACGGCTACCTTGTTACGACTT-3′. We performed PCR on extracted DNA samples using QUICK Taq HS DyeMix (TOYOBO Co., Ltd., Osaka, Japan). The PCR products were subjected to 1.2% agarose gel electrophoresis to detect a band of approximately 1,500 bp corresponding to 16S rRNA.

4.5. 16S rRNA Gene Amplicon Sequencing Analysis

For amplicon sequencing, libraries were prepared according to the Illumina 16S metagenomic sequencing library preparation protocols. 2.5 µl of microbial genomic DNA (5 ng/µL in 10 mM Microbial genomic DNA 2.5 µl (5 ng/µL in 10 mM Tris pH 8.5) was used for 1st PCR with the following primers: forward, 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTAHGGGRBGCAGCAG-3,′ Reverse 5′-GTCGTGGGCTCTCGAGAGATGTGTAGTAAGAGACAGGACTACHVGGGGTATCTAATCC-3′. PCR was performed using KAPA HiFi HotStart ReadyMix (KAPA Biosystems Inc., Washington, MA, United States) was used for PCR, and PCR products were analyzed using a Bioanalyzer DNA 1000 chip (Agilent Technologies LTD., Santa Clara, CA, United States). PCR products were purified using an Agencourt AMPure XP 60 ml kit (Beckman Coulter Co., Brea, CA, United States). Then, 2nd PCR was performed to construct a sequencing library following the Illumina 16S Metagenomic Sequencing Library Preparation Protocol, and the PCR products were purified and subjected to amplicon sequencing. Sample sequencing was performed using Illumina MiSeq (Illumina Inc., San Diego, CA, U.S.A.) and Paired-End Sequence data detected in each sample were stored in separate FASTAQ files for Read1 and Read2. Sequence data were analyzed using the Qiime2. First, demultiplexing and denoising were performed to remove adapter sequences, linker sequences, and primer sequences.

4.6. Microbial Population Analysis

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Before analysis, ASVs (Amplicon Sequence Variants) were generated by filtering, denoising, chimera check, and merging pair-reads of the sequence data according to the DADA2 workflow [8]. The Silva dataset was used for the 16S rRNA taxonomy database [9] and the V3-V4 region of the 16S rRNA was cut from the full-length sequence to adjust the dataset for analysis. The alpha diversity of each flora and beta diversity of the detected sites were examined. Kruskal-Wallis test was used for alpha diversity and evenness, and the observed features, Faith pd index, and Shannon index were used for beta diversity. Principal Coordinate Analysis (PCoA) was performed based on the distance and read count values in the phylogenetic tree of the detected fungi and PERMANOVA was used for the test.

5. Conclusions

Oral dysbiosis and the resulting bacteremia may be associated with the onset or progression of aortic valve disease.

Supplementary Materials: The supporting information can be downloaded at website of this paper posted on Preprints.org.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and the Ethical Guidelines for Medical and Health Research Involving Human Subjects issued by Japan Ministry of Health, Labor and Welfare and approved by the Ethics Committee of Dokkyo Medical University (Approval No. R－37－20J).

Informed Consent Statement: Written Informed consent was obtained from all participants. In cases where the patients themselves could not decide; we explained the details of the research to the legal guardian of the patient. Written informed consent has been obtained from the patients to publish this paper

Data Availability Statement: Authors declare that they have no competing interests. All data are available in the main text or the supplementary materials.

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