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Quantification and detection of bacteria from postoperative maxillary cyst by polymerase chain reaction

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Background/aims: Postoperative maxillary cyst (POMC) is known to occur as a delayed complication of radical maxillary sinus surgery, such as Caldwell-Luc surgery. The cyst gradually expands with no symptoms over a period of years, and then occasionally causes swelling and pain in the buccal region and/or the mucogingival fold. It is probable that bacterial infection affects the progression of POMC symptoms. The aims of this study were to determine the bacterial density and to examine the presence of 20 oral bacteria in POMC fluids.
Methods: POMC fluids (4 purulent, 2 mucous and 4 serous) were sampled from 10 subjects (aged 43–77 years). Bacterial quantification and detection were performed by real-time polymerase chain reaction (PCR) and nested PCR based on bacterial 16S rRNA genes, respectively.
Results: Bacterial DNA was detected in all samples and the average concentrations of bacterial DNA were 5.9 (purulent), 0.5 (mucous), and 0.7 (serous) ng/mg of sample. Twelve bacterial species, including anginosus streptococci, known to be associated with abscess formation, were detected in the purulent fluids, while two and five species were detected in the mucous and serous fluids, respectively.
Conclusion: Purulent fluids contained numerous bacteria of various types, thus suggesting that oral bacteria may cause symptoms such as pain in POMC with purulent fluids. Mucous and serous fluids also contained bacteria, although their numbers were small, thus suggesting an association between bacteria and progression of POMC.

Key words: 16S ribosomal RNA; cyst fluid; maxillary sinus; polymerase chain reaction; postoperative complications

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Postoperative maxillary cyst (POMC) is known to occur as a delayed complication of radical maxillary sinus surgery, such as Caldwell-Luc surgery. It is believed that POMC develops when the residual mucosa of maxillary sinus and mucous glands is trapped in the wound and the sinus ostia is obstructed (16, 22). The cyst gradually expands as a result of increased fluid retention and bone remodeling over a period of years, and occasionally causes swelling and pain in the buccal region and/or the mucogingival fold (11, 12). Although the cyst cavity is isolated from the nasal cavity by the cyst wall, which is covered with lining epithelium, it is probable that oral bacteria invade the obstructed cyst cavity through the teeth or periodontal pockets and cause the symptoms of POMC (34). Because the bottom of the sinus antrum is thin across the oral cavity and the cyst tends to expand toward the oral cavity by gravitation (10, 30), bone resorption of the bottom of sinus antrum of POMC sometimes occurs, resulting in vulnerability to bacterial invasion.

It has been estimated by culturing and molecular biological methods that more than 600 bacterial species inhabit the human oral cavity (21, 31, 35, 40, 49). Few bacteriological studies on POMC have been performed (17, 42), and thus the relationship between the presence of bacteria and the progression of POMC symptoms remains unclear.

The aims of this study were to determine the bacterial density of the cyst fluids by real-time polymerase chain reaction (PCR) and to examine the presence of 20 oral bacteria (Table 1) by nested PCR based on bacterial 16S rRNA genes. These bacteria were selected in this study since these species have frequently been isolated as putative pathogens from various oral diseases such as odontogenic infections, alveolar abscesses, periodontitis, and dental caries lesions (14, 23, 24, 37).

Material and methods

Subject population

Informed consent was obtained from each subject, and POMC fluids were sampled from 10 subjects (mean age, 57.2 ± 10.3 years; range, 43–77 years) (Table 2). Three of these subjects received antimicrobial therapy less than 2 weeks before sampling.

Collection of samples

Retained POMC fluids were collected using disposable sterilized 19-gauge needles attached to syringes and poured into sterilized vials. At the time of cystectomy or marsupialization, fluids were aspirated directly from the cysts. Samples were punctured from a disinfected area in the most prominent or fluctuant mucosal point overlaying the cyst. All samples were immediately transferred and stored at −20°C before extraction of genomic DNA.

DNA extraction

Each sample (5.0–9.9 mg) was immersed in 1 ml of sterilized distilled water and genomic DNA was extracted using a GFX Genomic Blood DNA Purification Kit (Amersham Bioscience, Piscataway, NJ) according to the manufacturer's instructions.

Real-time PCR

Quantitative real-time PCR amplification was performed with universal primers 357F and 907R (27), and iQ SYBR Green Supermix (Bio-Rad Laboratories, Richmond, CA), according to the manufacturer's instructions. The primer sequences were: 357F, 5′-CTC CTA CGG GAG

Table 1. Target bacteria and their species-specific primers

Species	Sequence (5′–3′)	Size (bp)	References
Anaerococcus sp.	GCG TGA TTT AGA AGG C ACG GGC GGT GTG TAC	980	(41)
Campylobacter rectus[a]	TTT CGG AGC GTA AAC TCC TTT TC TTT CTG CAA GCA GAC ACT CTT	598	(1)
Dialister pneumosintes	TTC TAA GCA TCG CAT GGT GC GAT TTC GCT TCT CTT TGT TG	1105	(6)
Eikenella corrodens	CGA TTA GCT GTT GGG CAA CTT ACC CTC TGT ACC GAC CAT TGT AT	410	(9)
Eubacterium saphenum	TCT ACT AAG CGC GGG GTG A ATA CCC GAT TAA GGG TAC	430	(13)
Finegordia magna	GCA TAA AAT CGT AGA AAC AC ACG GGC GGT GTG TAC	1200	(41)
Fusobacterium nucleatum	GAA GAA ACA AAT GAC GGT AAC AAC GTC ATC CCC ACC TTC CTC CT	705	(39)
Mogibacterium timidum	AAG CTT GGA AAT GAC GC CCT TGC GCT TAG GTA A	524	(13)
Peptoniphilus asaccharolyticus	ATG AAA ATC AAA CAG AAC C ACG GGC GGT GTG TAC	300	(41)
Peptostreptococcus anaerobius	GTA GTT AGC CTC CGA AA ACG GGC GGT GTG TAC	780	(41)
Peptostreptococcus micros	TCG GGA CAA CTA TAC AG ACG GGC GGT GTG TAC	380	(41)
Porphyromonas gingivalis[b]	GCG TAT GCA ACT TGC CTT AC GTT TCA ACG GCA GGC TGA AC	518	(46)
Prevotella intermedia	CGT GGA CCA AAG ATT CAT CGG TGG A CCG CTT TAC TCC CCA ACA AA	259	(33)
Prevotella nigrescens	GTG TTT CAT TGA CGG CAT CCG ATA TGA AAC CCA CGT CTC TGT GGG CTG CGA	828	(33)
Propionibacterium acnes[c]	AAG GCC CTG CTT TTG TGG ACT CAC GCT TCG TCA CAG	388	(15)
Slackia exigua	GCC AAG CGG CCT CGT CGA AG GCC GGC TTT AAG GGA TTC GCT CG	697	(13)
Streptococcus anginosus	ATG CAA TTG CAT CGC TAG T GCA GGC TTT GGA AAC TGT TTA ACT	445	(18)
Streptococcus constellatus	GTG CAA GAG CAT CAC TAC C GCA GGC TTT GGA AAC TGT TTA ACT	445	(18)
Streptococcus intermedius[d]	GTG CAA ATG CAT CAC TAC C GCA GGC TTT GGA AAC TGT TTA ACT	445	(18)
Streptococcus mutans	GGT CAG GAA AGT CTG GAG TAA AAG GCT A GCG GTA GCT CCG GCA CTA AGC C	282	(39)

[a]*In-silico* determination of the specificity, the primers may cross-react to *Campylobacter curvus*, *Campylobacter showae,* and *Campylobacter sputorum*.
[b]*In-silico* determination of the specificity, the primers may cross-react with *Porphyromonas gulae.*
[c]*In-silico* determination of the specificity, the primers may cross-react with *Propionibacterium* sp. LG and uncultured phylotypes (clones), i.e. lw29, Tc134–108, PE40, PE36, PE34, PE33PE30, PE27, PE22, PE21, PE20, PE15, PE13, PE12, PE06, 47 mm60, ACTINO8A, AT425_EubE10, DZ_D6, 1519, and PH-B24N.
[d]*In-silico* determination of the specificity, the primers may cross-react with *S. pneumoniae, S. pseudopneumoniae*, and *Streptococcus* sp. MGH.

Table 2. Clinical signs and conditions of subjects in this study

	Subjects									
	1	2	3	4	5	6	7	8	9	10
Age	50	50	48	43	66	58	53	62	65	77
Gender	M	M	M	M	F	M	M	F	M	F
Swelling	+	+	+	+	+	+	+	+	+	+
Pain	+	+	–	+	–	+	–	+	–	–
Antibiotic therapy[a]	–	–	–	+	–	+	–	–	+	–
Fluid state	Purulent	Purulent	Purulent	Purulent	Mucous	Mucous	Serous	Serous	Serous	Serous

[a]Application of antibiotic therapy (2 weeks before sampling) is designated as positive.

GCA GCA G-3′; and 907R, 5′-CCG TCA ATT CMT TTR AGT TT-3′. Real-time PCR amplification was performed in an iCycler (Bio-Rad Laboratories) programmed for 3 min at 95°C for initial heat activation, followed by 40 cycles of 15 s at 95°C for denaturation, 30 s at 55°C for annealing and 30 s at 72°C for extension. During the extension step, fluorescence emissions were monitored, and data were analyzed using iCycler iQ Software (Bio-Rad Laboratories). The genomic DNA of *Propionibacterium acnes* ATCC 6919 was used as a standard for quantitative analysis.

Nested PCR

In the first amplification, the 16S rRNA genes were amplified by PCR with universal primers 27F and 1492R (27) and Taq DNA polymerase (HotStarTaq Master mix; Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. The primer sequences were: 27F, 5′-AGA GTT TGA TCM TGG CTC AG-3′; and 1492R, 5′-TAC GGY TAC CTT GTT ACG ACT T-3′. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals, Ohtsu, Shiga, Japan) programmed for 15 min at 95°C for initial heat activation, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 55°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted PCR product with the universal primers was 1505 bp in length. In the second amplification (nested PCR), the first PCR products were used as templates and 20 bacteria were identified using species-specific primers based on 16S rRNA gene sequences (Table 1). In order to determine the specificity of the primers *in-silico*, blast searches were performed through the web site of the National Center for Biotechnology Information. PCR amplification was performed in a PCR Thermal Cycler MP (TaKaRa Biomedicals) programmed for 15 min at 95°C for initial heat activation, followed by 35 cycles of 1 min at 94°C for denaturation, 1 min at 58.5°C for annealing, and 1.5 min at 72°C for extension, and 10 min at 72°C for final extension. The predicted sizes of PCR products with species-specific primers are listed in Table 1.

PCR products were separated on 1% agarose gels (Certified Low Range Ultra Agarose, Bio-Rad Laboratories) stained with ethidium bromide and photographed under ultraviolet light. The sizes of PCR products were compared with a molecular size marker (Ready-Load 100-bp DNA Ladder, Invitrogen Corp., Carlsbad, CA), and confirmed to correspond to those listed in Table 1.

Data analysis

Fisher's exact probability tests and Tukey's tests were used to analyze significance. *P*-values of < 0.05 were considered statistically significant.

Results

Clinical signs of subjects and the state of cyst fluids are shown in Table 2. All patients had swelling and half had pain in the buccal region and/or mucogingival fold. Three patients received antibiotic therapy less than 2 weeks before sampling. Four of the fluids samples were purulent, two mucous and four serous.

The results of quantitative real-time PCR and nested PCR are shown in Table 3. Bacterial DNA was detected in all samples, and thus bacteria were proven to be present in all samples. The average quantity of bacterial DNA was 5.9 ± 2.1 (purulent), 0.5 ± 0.2 (mucous), and 0.7 ± 0.1 (serous) ng/mg of sample. Purulent fluids contained approximately 8–12 times more bacteria than mucous and serous fluids. Bacterial DNA was detected in the cyst fluids of the patients who received antibiotic therapy less than 2 weeks before sampling (Tables 2 and 3).

P. acnes was detected in nine samples, and its detection frequency was the highest among the bacterial species tested in this study. *Porphyromonas gingivalis*, *Streptococcus constellatus,* and *S. intermedius* were detected in five samples, Anaerococcus sp., *Fusobacterium nucleatum* and *Streptococcus anginosus* in three samples, *Campylobacter rectus*, *Dialister pneumosintes*, *Eikenella corrodens*, *Mogibacterium timidum,* and *Peptostreptococcus micros* in two samples, and *Slackia exigua* in one sample. Anaerobic gram-positive cocci such as *Peptostreptococcus* species, which is frequently detected in oral maxillofacial infections (24), were detected in small numbers in this study. The other seven species tested for were not detected in this study.

In the purulent fluids, 12 bacterial species including anginosus streptococci, which is reported to be associated with abscess formation (25, 26), were detected, while two and five species were detected in the mucous and serous fluids, respectively. *P. gingivalis* was detected with a high frequency (three of four cases) in the serous fluids. In eight cases, two species and/or more than two species were detected. In two cases (one mucous and one serous), *P. acnes* was the only species detected in this study.

In *in-silico* determination of the specificity of the primers used in this study, the primers of *P. gingivalis* were found to cross-react with *Porphyromonas gulae*; those of *P. acnes* to cross-react with Propionibacterium sp. LG and uncultured phylotypes (clones), i.e. lw29, Tc134–108, PE40, PE36, PE34, PE33PE30, PE27, PE22, PE21, PE20, PE15, PE13, PE12, PE06, 47 mm60, ACTINO8A, AT425_EubE10, DZ_D6, 1519, and PH-B24N; those of *C. rectus* to cross-react with *Campylobacter curvus, Campylobacter showae,* and *Campylobacter sputorum*; and those of *S. intermedius* to cross-react with *Streptococcus pneumoniae*, *Streptococcus pseudopneumoniae,* and *Streptococcus* sp. MGH.

Discussion

In this study, bacterial DNA was detected by quantitative real-time PCR in all samples (Table 3). A low bacterial detection frequency (< 50%) has been reported in POMC fluids (17, 42), and the discrepancy

Table 3. Total bacterial DNA and detected bacterial species

	Purulent				Mucous		Serous				
	1	2	3	4	5	6	7	8	9	10	
	7.9 [b]	7.5	4.7	3.5	0.4	0.6	0.6	0.7	0.9	0.8	No. of positive samples
Universal[a]	(5.9 ± 2.1[b, c, d])				(0.5 ± 0.2)		(0.7 ± 0.1)				10
Propionibacterium acnes	+	+	+	–	+	+	+	+	+	+	9
Porphyromonas gingivalis	–	–	+	–	+	–	+	+	+	–	5
Streptococcus constellatus	+	+	+	+	–	–	+	–	–	–	5
Streptococcus intermedius	+	+	+	+	–	–	+	–	–	–	5
Anaerococcus sp.	+	+	+	–	–	–	–	–	–	–	3
Fusobacterium nucleatum	+	+	+	–	–	–	–	–	–	–	3
Streptococcus anginosus	+	+	+	–	–	–	–	–	–	–	3
Campylobacter rectus	+	+	–	–	–	–	–	–	–	–	2
Dialister pneumosintes	+	+	–	–	–	–	–	–	–	–	2
Eikenella corrodens	+	+	–	–	–	–	–	–	–	–	2
Peptostreptococcus micros	+	+	–	–	–	–	–	–	–	–	2
Mogibacterium timidum	+	–	–	+	–	–	–	–	–	–	2
Slackia exigua	–	–	–	–	–	–	–	+	–	–	1
Eubacterium saphenum	–	–	–	–	–	–	–	–	–	–	0
Finegordia magna	–	–	–	–	–	–	–	–	–	–	0
Peptostreptococcus anaerobius	–	–	–	–	–	–	–	–	–	–	0
Peptoniphilus asaccharolyticus	–	–	–	–	–	–	–	–	–	–	0
Prevotella intermedia	–	–	–	–	–	–	–	–	–	–	0
Prevotella nigrescens	–	–	–	–	–	–	–	–	–	–	0
Streptococcus mutans	–	–	–	–	–	–	–	–	–	–	0
No. of positive bacterial species	11	10	7	3	2	1	4	3	2	1	

[a] Quantification of total bacterial DNA by real-time PCR with universal primers of 16S ribosomal RNA genes.
[b] ng per mg of sample.
[c] Mean values ± standard deviations.
[d] Significantly different ($P < 0.05$) from mucous and serous sample.

could be because previous reports depended on culturing methods and fastidious bacteria were dominant in POMC fluids. In our study, however, the use of molecular biology made it possible to detect bacteria from mucous and serous cyst fluids despite their small numbers.

P. acnes was detected in nine samples and was the most frequently detected bacterium (Table 3). *P. acnes* is an anaerobic gram-positive rod that is considered to be an indigenous bacteria of skin as well as oral and intestinal mucosa. However, the species has been reported to have pathogenicity based on superantigenicity and mitogen activity of T cells (2, 8, 19). *P. acnes* has been recently detected in various diseases, such as alveolar abscess, sinusitis, osteomyelitis, meningitis, noma, endocarditis, septicemia, hepatitis granuloma, facial acne, and abscess of orbit (5, 28, 29) as well as in opportunistic infection (5).

P. gingivalis, detected in five samples in this study (Table 3), is an obligate anaerobic gram-negative rod, and is reportedly associated with the pathogenicity of periodontitis, alveolar abscesses, and oral infections (7, 23). *P. gingivalis* possesses high proteolytic activity to degrade proteins in periodontal tissues and gingival fluids due to expression of various peptidases, such as gingipain (36, 45). *P. gingivalis* also produces lipopolysaccharide and cytotoxic metabolites such as ammonia, butyrate, and propionate (32, 44). Therefore, *P. gingivalis*, detected in POMC fluids, may be associated with POMC inflammation. *P. gingivalis* was detected with a high frequency in the serous fluids in this study, thus suggesting that the properties of serous fluids resembling those of serum, which is rich in protein and neutral in pH (3), may provide a suitable environment for *P. gingivalis* to survive (43).

S. constellatus and *S. intermedius*, detected in five samples in this study (Table 3), and *S. anginosus,* detected in three samples in this study (Table 3), are designated as the anginosus group of streptococci. The anginosus groups are facultative anaerobic gram-positive cocci and are indigenous bacteria in humans. It has been reported that they possess pathogenicity to form abscesses (4, 24, 48) based on tolerance to polymorphonuclear leukocytes (47), cellular components such as capsules (20), and extracellular enzymes such as hyaluronidase (38). It has also been reported that abscess formation by the anginosus group is enhanced by interaction with other bacteria, such as *F. nucleatum*, because its heat-resistant extracellular materials may enhance the pathogenicity of the anginosus group (25, 26). The growth of *S. constellatus* was enhanced by the presence of *E. corrodens* (4). In this study, particularly in the purulent samples, the anginosus group of streptococci was frequently detected with *F. nucleatum* and *E. corrodens* (Table 3). These results support previous reports, and suggest that bacterial interaction may be a symptom of POMC.

In *in-silico* determination of the specificity of the primers used in this study, the primers of *P. gingivalis, P. acnes, C. rectus,* and *S. intermedius* were found to cross-react with other established species and uncultured phylotypes. Thus, it is possible that some uncultured phylotypes which resemble established species, such as *P. acnes,* were frequently detected by PCR in this study, and that these phylotypes may relate to the pathogenicity of POMC, although further studies on the taxonomy of these phylotypes and their pathogenic roles are required.

Purulent fluids of POMC contained numerous bacteria of various types in this study (Table 3), suggesting that the bacteria in the purulent fluids are associated with POMC symptoms such as pain. Pain was the most common clinical manifestation in purulent cases in this study (Table 2). On the other hand, mucous and serous fluids also contained bacteria, although their numbers were small (Table 3), suggesting that the increase in internal pressure of POMC resulting from increases in the amount of fluid is closely related to POMC symptoms, although bacteria may be related to the progression of POMC symptoms.

It has been reported that various types of bacteria are detected in oral maxillofacial infections and periodontal diseases, and many efforts have been made to determine the pathogenicity of these bacteria. Nevertheless, the relationship between bacteria and POMC symptoms has not been widely investigated. Our results suggest that bacteria, frequently detected in the oral cavity, were present in the fluids of POMC, and that these bacteria were partially responsible for the progression of POMC symptoms, although further studies on the presence of bacterial species other than the 20 species selected in this study and their etiologic roles are required. PCR methods are effective in detecting fastidious bacteria in maxillofacial infections and these methods will assist in conventional bacteriological examination, including culturing methods.

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