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Bacteriological findings in radicular cyst and keratocystic odontogenic tumor fluids from asymptomatic patients

Daniela Scalas[1], Janira Roana[1], Paolo Boffano[2], Narcisa Mandras[*], Cesare Gallesio[2], Mario Amasio[2†], Giuliana Banche[1], Valeria Allizond[1], Anna Maria Cuffini[1]

[1]Department of Public Health and Pediatrics, University of Torino, Via Santena 9, 10126, Turin, Italy; [2]Division of Maxillofacial Surgery, Head and Neck Department, University of Torino, Corso Dogliotti 14, 10126, Turin, Italy

† He passed away on 30 September 2012. He left us in a whisper. He left us here to cry ... but his ideas still live with us.

* **Corresponding author**
Dr. Narcisa Mandras
Department of Public Health and Pediatrics
Microbiology Section, University of Torino
Via Santena 9, 10126 Turin, Italy
Phone: +39/0116705645
Fax: +39/0112365645
e-mail: narcisa.mandras@unito.it

ABSTRACT

Objective. In this study the potential presence of bacteria in radicular cyst (RC) and keratocystic odontogenic tumor (KCOT) fluids from clinically asymptomatic patients was investigated.

Materials and methods. Cyst fluids were collected by needle aspiration from 16 patients with asymptomatic osteolytic lesions (10 RCs and 6 KCOTs) undergoing surgery. All samples were tranferred into tubes containing pre-reduced transport medium, delivered to the microbiology laboratory and processed within 1 hour. The cysts, surgically enucleated, were sent for standard histopathological examination. Cyst fluid samples were cultured on selective and differential media in anaerobic (for about 2 weeks) and aerobic (for 24-48 hours) conditions to detect viable microorganisms. After incubation, the colonies were counted, Gram-stained and identified by biochemical tests.

Results. Cultures were positive for the presence of bacteria in 15 (9 RCs, 6 KCOTs) out of 16 cases. RCs and KCOTs generally yielded low bacterial counts (10^2 - 10^4 CFU/ml) and were predominantly colonized by obligate anaerobes (64%), whereas less commonly by facultative anaerobes (36%). No significant differences in the detection frequencies of obligate and facultative anaerobes were evidenced between RCs and KCOTs. *Propionibacterium acnes* was the most common obligate anaerobe recovered both in RC and KCOT fluids. Among facultative anaerobes, *Gemella morbillorum* was more frequently isolated in KCOTs, whereas *Staphylococcus spp.* in RCs.

Conclusions. Bacteria may be present and persist within fluids of clinically asymptomatic jaw cystic lesions. The influence of bacteria and latent bacterial

infection within cystic jaw lesions should be reconsidered in odontogenic cyst progression.

Keywords: bacteria, radicular cyst, keratocystic odontogenic tumor, asymptomatic patients

Running title: Bacteria and odontogenic jaw cysts

1. Introduction

Odontogenic cysts of the jaws include various pathological entities, all arising from the epithelial residues of the tooth-forming organ. These lesions have been traditionally classified into two groups: developmental or non- inflammatory cysts ("odontogenic keratocysts", dentigerous, gingival cysts, etc.) and inflammatory cysts (radicular, residual, paradental cysts). Among them, radicular cysts (RCs) and "odontogenic keratocysts" draw special attention. RCs are the most common bone-destroying lesions and are associated with the root apex of a tooth with necrotic pulp. They comprise about 56% of all the cysts affecting the human jaws, followed by dentigerous and "odontogenic keratocysts" (1, 2). The inflammatory dental periapical granuloma is considered to be the origin of RC formation. Humoral and cell-mediated reactions have been implicated in RC pathogenesis, whereas inflammatory cytokines in the proliferation of epithelial cell rests (3, 4). On the other hand, "odontogenic keratocysts" are benign uni-or multicystic intraosseous neoplastic lesions with the potential for aggressive, infiltrative behavior and genetic factors are thought to play a major role in their etiology (5). They comprise about 11% of all jaw cysts and are characterized by high recurrence rate (range 3-60%) and association

with nevoid basal cell carcinoma syndrome (2). The World Health Organization in 2005, based on behavior, histology, and genetics, reclassified "odontogenic keratocysts" with parakeratinized stratified squamous epithelium as keratocystic odontogenic tumors (KCOTs) (6). Through the years, there have been conflicting reports regarding the presence of microorganisms in odontogenic cysts and their role in cyst pathogenesis. Some authors suggest that bacteria are not found in RCs and do not normally penetrate into the lumen, unless as a result of a secondary infection (7, 8). Through analysis of fluids and explants media from RCs and KCOTs without any evidence of overt infection, Meghji *et al.* found higher level of endotoxin in RCs than in KCOTs, but no obligate or facultative anaerobic bacteria within cyst fluids (7). In histological analysis of bacterial status in root-filled teeth with osteolytic lesions exposed to oral environment, Ricucci and Bergenholz found bacteria in the pulp canals and in the immediate region of the apical foramena, but not in the cyst walls or lamina (8). However, other studies have shown that infected odontogenic cysts do indeed contain microorganisms and may not be sterile (9, 10). In a microbiological study of the fluids of infected jaw cysts, predominantly radicular and residual, Iatrou and co-workers (9) reported that approximately 89% of bacteria isolated from the fluid of infected cysts were obligate anaerobes, while only 10% corresponded to aerobes or facultative anaerobes. Gram positive anaerobic cocci were the most frequent bacterial group, followed by Gram negative anaerobic rods and aerobic cocci. Since no exhaustive results are available in this research-area, we investigated the potential presence of bacteria in cystic fluid from non-ruptured RCs by comparing the results with corresponding findings in non-inflammatory lesions like KCOTs, to give quantitative and qualitative information on the microorganism content within the fluids of clinically asymptomatic cystic lesions of the jaws.

2. Materials and methods

2.1. Patients

The study enrolled 16 patients with asymptomatic osteolytic lesions (6 females and 10 males, 34–82 years of age), which were clinically and radiographically provisionally diagnosed as odontogenic cysts during routine examination at the Division of Maxillofacial Surgery, Head and Neck Department, University of Torino, Turin, Italy. All patients were in general good health, without any clinical sign or symptoms of infection (pain, fever, swelling) at the time of surgery. Age, sex, type of cyst lesions, lesion maximum diameter, and lesion site of enrolled patients were recorded. Patients participating in this study gave their informed written consent.

2.2. Cyst fluids

Cyst fluids from 16 non-ruptured odontogenic cysts were aspirated (other samples contaminated with blood were not enrolled in the study). Initial local disinfection of oral mucosa with chlorexidin was performed before each surgical intervention. Surgical interventions were performed under local anesthesia with two 1.8-ml capsules of 2% mepivacaine containing 1:100,000 adrenaline. A buccal envelope mucoperiosteal flap was raised in all cases. A further local disinfection of the exposed bone was performed by chlorexidine and new sterile surgical instruments were used hereafter, in order to avoid possible contamination. Then, the osteotomy necessary to visualize the cystic lesion was performed using a no. 8 tungsten carbide round bur mounted on a high-speed handpiece, paying attention not to damage the cystic walls. The integrity of the cystic walls was checked in each case: none of the 16 studied cysts had damaged walls. During oral surgery practice various cysts had

damaged or perforated cyst walls, but they were not enrolled in the study, as they did not meet the inclusion criteria.

The cystic fluid was withdrawn by a sterile syringe through the cyst walls, paying attention not to get in contact with the bone or with eventually protruding tooth apexes. The aspirate was injected, without introducing air, in a sterile test tube with 1 ml of pre-reduced transport medium (BBL™ Port-A-Cul™, Becton Dickinson Italia S.p.a., BD, Buccinasco, Milan, Italy) to ensure specimen viability, delivered to the microbiology laboratory and processed within 1 hour after collection. The quantity of aspirate was recorded: the aspirated volume ranged from 0.5 to 1.5 ml. Finally, cysts were totally enucleated and sent for histopathological examination.

2.3. Histopathological analysis

Histopathological examination of the specimens was performed by hematoxylin-eosin staining. Criteria of diagnosis of RCs were defined as the presence of multilayered nonkeratinized squamous epithelium, whereas KCOTs were diagnosed when a characteristic lining of parakeratinized stratified squamous epithelium (eventually together with thickened squamous epithelium, daughter cysts, and budding proliferation of the epithelium) was found.

2.4. Microbiological analysis

Isolation and identification of bacterial species in cyst fluid samples were made by microbiological culture techniques, as DNA-based molecular methods, despite their higher microbial sensitivity and specificity, do not differentiate between viable and non-viable microorganisms and/or contaminants. This technique enabled us to search

for all the microorganisms present in cyst fluid samples, including unexpected bacterial species.

Cyst fluids were cultured to quantify the total microbiota and the number of obligate and facultative anaerobic bacterial strains. All samples were vortexed for 30 seconds and diluted 1:10 in normal saline (0.9% NaCl). Further serial dilutions (10^{-2} to 10^{-3}) were made in normal saline and 100 µl were plated on agar plates. The bacteria were quantified on selective and differential media suitable for the growth of obligate and facultative anaerobic bacteria: Brain Heart Agar (BHA, BD) to determine the number of facultative anaerobic strains, Plate Brucella Agar (BD) to determine the total number of anaerobic (obligate and facultative) bacterial strains and Schaedler CNA agar plus 5% blood (BD) and Schaedler Kanamicina-Vancomicina agar plus 5% blood (BD) for Gram positive and Gram negative obligate anaerobes, respectively. All plates were incubated at 37°C, BHA plates for 24–48 hours under aerobic conditions, and the other plates for about 2 weeks under anaerobic conditions in an anaerobic system (Gaspak EZ anaerobe pouch system kit, BD). All aerobic cultures were examined at 24 and 48 hours, whereas anaerobic cultures were kept for at least two weeks and examined for growth every 3 days. After incubation, the colonies were counted and Gram-stained. The microbe counts were reported as colony-forming units/ml (CFU/ml). To identify microorganisms, biochemical tests were performed with commercial API Systems (BioMérieux, Rome, Italy) according to the manufacturer's instructions: API Staph for aerobic bacteria, API Strep for anaerobic facultative bacteria, and API 20 A for anaerobic obligate bacteria (11, 12).

2.5. Statistical analysis

Fisher's exact probability tests, chi-squared tests, and independent Student's t-tests were used to analyze significance. P-values of 0.05 or less were considered statistically significant. All statistical analyses were performed using the Graphpad Prism version 5.00 for Windows (Graphpad Software, San Diego, CA, USA).

3. Results

Data of the study population are presented in Table 1. Mean age of the patients was 53 years (range, 34 to 82 years; median, 50; Standard Deviation, 15.80). Six patients were females and 10 males. Mean maximum diameter of odontogenic lesions was 2.59 cm (range, 1.5 to 5 cm; median, 2; SD, 1.05). Mandible was involved in 6 cases, whereas 10 cysts were located in the maxilla.

The clinical and radiographic diagnosis of the lesions as radicular cysts and keratocystic odontogenic tumors was confirmed by histopathological examination: the definitive diagnosis of 10 RCs and 6 KCOTs was performed.

Cultures were positive for the presence of bacteria in 15 (9 RCs, 6 KCOTs) out of 16 cases. Total viable counts in positive cyst fluid samples ranged from 10^2 to 10^4 CFU/ml. A comparable mean total viable count both for facultative anaerobes (4.2 x 10^3 CFU/ml in KCOTs *vs.* 2.76 x 10^3 CFU/ml in RCs), and for obligate anaerobes (7.96 x 10^3 CFU/ml in KCOTs *vs.* 8.14 x 10^3 in RCs) was found. Statistical analysis failed to demonstrate significant differences between the RC and KCOT fluid samples examined ($P > 0.05$). The bacterial distribution in each sample is shown in Table 2: the mean number (± SD) of bacterial species per sample was 2.06 (±0.93). From all positive cyst fluid samples, a total of 12 bacterial strains were isolated. Eight strains (64%) were obligate anaerobes, and four (36%) were facultative anaerobes. Obligate anaerobes in RC samples accounted for 31% of the total isolates

versus 33% in KCOTs. Facultative anaerobes accounted for 21% of the total isolates in RCs *versus* 15% in KCOTs. These differences were not statistically significant ($P > 0.05$). Among obligate anaerobes, *Propionibacterium acnes* was the most common isolate both in RC (9%) and KCOT fluids (12%). Anaerobic Gram positive cocci such as *Peptostreptococcus spp.*, which are frequently found in oral maxillofacial infections, were detected with a higher frequency in KCOT (6%) than in RC (3%) samples. The frequency of detection of *Actinomyces naeslundii*, a Gram positive bacterial species usually involved in periodontal disease, was lower in KCOTs (3%) than in RCs (6%). *Porphyromonas asaccharolytica* was only identified in KCOTs (3%), whereas *Prevotella spp*. and *Veillonella parvula* were recovered both in RCs and KCOTs at the same rate (3%). Among the facultative anaerobe group, *Gemella morbillorum* was the most frequently bacterium encountered in KCOTs (9%). *Leuconostoc spp*. was recovered both in RCs and in KCOTs at a rate of 6% and 3%, respectively. *Staphylococcus spp*. was detected with a higher frequency in RC cyst fluids (12%), whereas *Staphylococcus saccharolyticus* was found both in RCs and in KCOTs at the same rate (3%). None of these results proved to be statistically significant ($P > 0.05$).

4. Discussion

Up to now, it is believed that cystic lesions of the jaws may be usually assumed to be sterile and generally clinically asymptomatic unless they are secondarily infected (4, 7). Nevertheless, few bacteriological studies on cystic fluids of odontogenic lesions in asymptomatic patients have been performed. In this study the bacterial content of cyst fluids from asymptomatic RCs and KCOTs was examined by microbiological culture techniques, to provide a thorough quantitative measure of viable

microorganisms in cyst fluid samples. It has to be underlined that microbiological culture techniques are still considered valuable tools for quantitatively detecting only living microorganisms. In fact, despite the advent of more sensitive DNA-based molecular methods that search for pre-selected target species, culture techniques have the advantage of being able to identify a wide variety of bacterial species, including unexpected or new species and to provide a quantitative measure of all the viable cultured microorganisms. All the cysts were clinically considered as uninfected and their fluids were cultured for extended incubation time (about 2 weeks) in anaerobic and aerobic conditions to detect viable microorganisms. This procedure allowed recovery of slow-growing anaerobes such as *Actinomyces* and *Propionibacterium* species.

Bacteria were found in 15 out of 16 asymptomatic odontogenic lesions (93.75%). RCs and KCOTs generally yielded low bacterial counts (range, 10^2 to 10^4 CFU/ml). Comparable mean total bacterial counts both for obligate and facultative anaerobes were observed between RC and KCOT fluid samples (Fig. 1). RCs and KCOTs were predominantly colonized by obligate anaerobic bacteria (64%), whereas facultative anaerobic bacterial species were less common (36%). No significant differences in the frequency of detection of obligate and facultative anaerobes between RCs and KCOTs were seen ($P > 0.05$). Overall, *P. acnes*, a Gram positive rod considered to be an indigenous of skin, oral cavity and intestinal mucosa, was the most frequently detected obligate anaerobe both in RC and KCOT fluids (Table 2). This bacterial species has been reported to have pathogenicity based on superantigenicity and mitogen activity of T cells and has been recently detected in various diseases, such as alveolar abscess, sinusitis, osteomyelitis, meningitis, endocarditis, septicemia, hepatitis granuloma, facial acne, and abscess of orbit as well as in opportunistic

infection (13, 14). Moreover, *P. acnes* is capable of surviving *in vitro* for as long as 8 months under anaerobic conditions without subculture, suggesting that it could also survive in human tissues at low oxidation potentials and damage them by its toxic metabolites (13, 15). Among the facultative anaerobe group, *G. morbillorum*, a slow-growing Gram-positive bacterium, was the most prevalent species in KCOT fluid samples, and it was found in combination with *P. acnes* in 3 out of 6 KCOT cases (Table 2). On the contrary, in addition to putative periodontal pathogens RCs were more frequently colonized by *Staphylococcus spp*., a clinically important pathogen, compared to KCOTs (Table 2).

In summary and within the limitations of this study, our data indicate that bacteria may be present in cyst fluids from asymptomatic RCs and KCOTs. In addition, it seems possible that some anaerobic bacterial strains could also multiply and persist within clinically asymptomatic cystic lesions of the jaws. These findings are in disagreement with those reported in 1996 by Meghji *et al*. (7), who found that all of the cyst fluids from symptomless RCs, KCOTs and follicular cysts were microbiologically sterile during the 72-h culture period, despite they detected endotoxin in RC fluids and, at a lower level, in KCOTs and follicular cysts. Based on these results, they suggested that endotoxin released by anaerobes in the infected necrotic tooth pulp induces inflammation around the apical area and has a major initiating role in the RC pathogenesis, without giving further explanations on the absence of bacteria within cyst fluids (7). It has to be underlined that since then, all the literature has defined cyst fluids as microbiologically sterile. In our opinion, this discrepancy may result more likely from differences in methodologies, including microbiological techniques and, most of all, incubation time, as many of the anaerobic species require extended anaerobic incubation for growth. Bearing in mind

the substantial etiological and pathogenetic differences between RCs and KCOTs, a positive bacterial detection in KCOTs was surely an unexpected interesting result. However, even if a contamination cannot be totally excluded in our study, we think that the different frequency of detected bacterial species in radicular cysts and KCOTs could be suggestive for an absence of contamination that would provide similar results. Presumably, it is possible that because of their keratinized lining, bacteria could proliferate within the cavities of KCOTs without causing clinical symptoms.

In conclusion, the influence of bacteria and latent bacterial infection within cystic jaw lesions should be reconsidered in odontogenic cyst progression. In view of this, further investigations in large number of samples could be useful to improve our understanding of the possible role of long-term persisting bacteria in cyst enlargement and progression. Furthermore, a better understanding of microorganism content in asymptomatic jaw cyst lesions should also aid in establishing a correct preoperative antibiotic coverage during oral surgery practice.

Conflict of interest The authors declare that they have no conflict of interest.

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Table 1. Study population

Patients	Age (years)	Sex	Lesion type	Lesion diameter (cm)	Lesion site
1	34	F	RC [a]	3	Mandibular body
2	42	M	RC	3	Mandibular symphisis
3	47	M	RC	2	Maxilla
4	51	F	RC	4	Maxilla
5	58	F	RC	2	Maxilla
6	61	M	RC	1.5	Maxilla
7	82	F	RC	2	Maxilla
8	34	M	RC	2	Maxilla
9	39	F	RC	2.5	Maxilla
10	32	M	RC	4	Maxilla
11	78	F	KCOT [b]	1.5	Mandibular angle
12	50	M	KCOT	2	Maxilla
13	50	M	KCOT	5	Mandibular angle
14	67	M	KCOT	2	Maxilla

15	74	M	KCOT	3.5	Mandibular angle
16	49	M	KCOT	1.5	Mandibular angle

[a] RC = radicular cyst

[b] KCOT = keratocystic odontogenic tumor

Table 2. Distribution of bacterial species in radicular cyst (RC) and keratocystic odontogenic tumor (KCOT) cyst fluid samples

	RCs										KCOTs						
Patients	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	***Positive samples (No.)***
Obligate anaerobes																	
Actinomyces spp.	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	**1**
Actinomyces naeslundii	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	+	**3**
Peptostreptococcus spp.	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-	-	**3**
Porphyromonas asaccharolytica	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	**1**
Prevotella spp.	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	**2**
Propionibacterium spp.	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	**2**
Propionibacterium acnes	-	+	-	-	+	+	-	-	-	-	+	+	-	+	-	+	**7**
Veillonella parvula	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	**2**
Facultative anaerobes																	
Gemella morbillorum	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	-	**4**
Leuconostoc spp.	-	-	-	-	-	-	-	+	+	-	-	-	+	-	-	-	**3**
Staphylococcus spp.	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	**3**
Staphylococcus saccharolyticus	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	-	**2**
Positive bacterial species/patient (No.)	**0**	**2**	**2**	**2**	**2**	**3**	**2**	**1**	**1**	**2**	**2**	**2**	**3**	**4**	**3**	**2**	