Enrichment of Multilocus Sequence Typing Clade 1 with Oral *Candida albicans* Isolates in Patients with Untreated Periodontitis

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This study investigated the prevalence and cell density of *Candida* species in periodontal pockets, healthy subgingival sites, and oral rinse samples of patients with untreated periodontitis. Twenty-one periodontitis patients underwent sampling at two periodontitis sites, and 19/21 of these patients underwent sampling at one periodontally healthy site. Both paper point and curette sampling techniques were employed. The periodontitis patients and 50 healthy subjects were also sampled by oral rinse. *Candida* isolates were recovered on CHROMagar Candida medium, and representative isolates were identified. *Candida* spp. were recovered from 10/21 (46.7%) periodontitis patients and from 16/50 (32%) healthy subjects. *C. albicans* predominated in both groups and was recovered from all *Candida*-positive subjects. *Candida*-positive periodontitis patients yielded *Candida* from periodontal pockets with average densities of 3,528 and 3,910 CFU/sample from curette and paper point samples, respectively, and 1,536 CFU/ml from oral rinse samples. The majority (18/19) of the healthy sites sampled from periodontitis patients were *Candida* negative. The 16 *Candida*-positive healthy subjects yielded an average of 279 CFU/ml from oral rinse samples. *C. albicans* isolates were investigated by multilocus sequence typing (MLST) to determine if specific clonal groups were associated with periodontitis. MLST analysis of 31 *C. albicans* isolates from periodontitis patients yielded 19 sequence types (STs), 13 of which were novel. Eleven STs belonged to MLST clade 1. In contrast, 16 *C. albicans* isolates from separate healthy subjects belonged to 16 STs, with 4 isolates belonging to clade 1. The distributions of STs between both groups were significantly different ($P = 0.04$) and indicated an enrichment of *C. albicans* isolates in periodontal pockets, which warrants a larger study.

The periodontium is composed of the gingivae, periodontal ligament, root cementum, and alveolar bone. In normal healthy gingivae, the free gingival margin and the tooth surface are in close proximity to each other, leaving very little space for microbial colonization (13). Periodontitis is an infection of the oral gingival tissue that is caused by a combination of microorganisms commonly found in dental plaque, such as streptococci, staphylococci, fusobacteria, *Porphyromonas* species, *Campylobacter* species, actinobacteria, and many others (30, 37). As periodontitis manifests, the gingival margin becomes enlarged, causing the gingival tissue to detach from the tooth, resulting in the formation of periodontal pockets. This coincides with the spread of infection and an associated inflammatory response leading to the irreversible destruction of the periodontal ligament and alveolar bone. As the disease progresses, the depth of these periodontal pockets (probing pocket depth) increases, and bleeding and/or suppuration upon the probing of periodontal tissues also occurs (11). The buildup of dental plaque, the development of dental caries, or denture wearing is a risk factor for the development of periodontitis, as are particular systemic conditions that affect host immune or inflammatory responses (15, 28, 36). The treatment of periodontitis consists of the mechanical cleaning of teeth and the debridement of the associated diseased tissue, followed by improved dental hygiene.

Candida species are commensal yeasts and opportunistic pathogens that reside on mucosal surfaces and can cause oropharyngeal infection albeit usually in immunodeficient individuals, those with severe underlying diseases, and upper denture wearers. In healthy oral carriers, *Candida* spp. typically reside on the buccal mucosa, tongue, and palate and in the saliva. *Candida* species have been isolated from 40 to 60% of healthy mouths (29), although they are rarely found in subgingival sites in patients with good oral health (35). *Candida* species have frequently been isolated from periodontal pockets; however, their role, if any, in the etiology of periodontitis remains to be elucidated (5, 8, 27, 30, 35, 37). Several previous studies investigated the prevalence and possible role of *Candida* species in periodontitis, all of which identified *C. albicans* as the *Candida* species most frequently isolated from periodontal pockets (3, 9, 19, 20, 31, 35). Several of those studies also detected the presence of other *Candida* species, including *Candida glabrata*, *Candida dubliniensis*, *Candida tropicalis*, *Candida guilliermondii*, and *Candida parapsilosis*, in the periodontal pockets of patients with periodontitis; however, it should be noted that two of those studies involved patients who also had insulin-dependent diabetes, who are prone to *Candida* colonization and infection (19, 31, 38). Urzúa et al. (35) reported previously that patients with chronic periodontitis had a significantly higher level of colonization with *Candida* at subgingival sites than periodontally healthy individuals. However, currently, it is difficult to attribute a definitive role of *Candida* species in periodontitis, as studies reporting the relative abundance of *Candida* in periodontal pockets

are mostly lacking. Furthermore, questions remain with regard to the optimal subgingival sampling technique. Previous clinical investigations comparing paper point and curette sampling methods noted significant differences between samples gained from the same site by employing these sampling techniques (7, 26). To date, there have been no investigations combining both paper point and curette sampling techniques in the investigation of the isolation of yeast species from periodontal sites.

Several previous studies investigated the genetic relatedness of *C. albicans* isolates recovered from the periodontal pockets, gingival sulci, and oral mucosae of patients with periodontitis by using molecular typing techniques such as random amplified polymorphic DNA (RAPD) fingerprinting, electrophoretic karyotyping, and ABC genotyping, with the latter technique being based on the presence or absence of an intron in the 25S ribosomal DNA (rDNA) (1, 24, 32, 33). Pizzo et al. (24) previously used electrophoretic karyotyping to show that some *C. albicans* genotypes are unique to subgingival isolates, suggesting some adaptation to this environment. In contrast, Barros et al. (1) identified a genetically homogenous population of *C. albicans* strains in the oral cavities of patients with periodontitis by using RAPD. However, RAPD can be affected by a multitude of variables, its reproducibility is poor, and its resolving power is significantly lower than those of targeted, species-specific DNA comparison methods such as multilocus sequence typing (MLST) (34). Another study revealed that 66/128 (51.6%) subgingival *C. albicans* isolates recovered from a group of 11 diabetic patients with periodontitis belonged to ABC genotype B, and the remaining isolates belonged to ABC genotype A. However, it is important to note that diabetic patients are prone to oral candidiasis. Furthermore, the discriminatory power of ABC genotyping is poor, as only three types can be discriminated by using this technique (32).

In the past decade, advances in DNA sequencing technologies have enabled informative and detailed population structure analyses of *Candida* species to be undertaken, particularly following the establishment of species-specific MLST schemes. The application of MLST to yeast species enables the direct comparison of concatenated DNA sequence data and the resulting sequence types (STs) from distinct groups of isolates, for example, those recovered from distinct anatomical sites, geographical locations, or patient cohorts.

Previous MLST-based population biology analyses were carried out on both *C. albicans* and *C. dubliniensis*. The population structure of *C. albicans* consists of 17 different MLST clades to which 97% of *C. albicans* isolates can be assigned (23). These isolates can also be distinguished by ABC genotyping, and the proportions of isolates of the A, B, and C genotypes can differ significantly between MLST clades. The population structure of *C. albicans* can also be analyzed by using an algorithm that is based upon related sequence types (BURST). This analysis is based on the allelic profiles defined by MLST and enables isolates to be grouped into clonal complexes (CCs) as well as predictions of putative founding genotypes. The *C. dubliniensis*-specific MLST technique was developed by researchers at this laboratory and has shown that the population of *C. dubliniensis* is composed of three closely related clades that can be distinguished further on the basis of the DNA sequence of the internal transcribed spacer (ITS) region of the rDNA operon.

The main purpose of the present study was to investigate the prevalence and relative abundance of *Candida* species in peri-odontal pockets, healthy subgingival sites, and oral rinse samples of patients with untreated periodontitis. Isolates of the two most prevalent species recovered, *C. albicans* and *C. dubliniensis*, were subjected to MLST analysis. *C. albicans* isolates recovered from periodontitis patients were compared with oral carriage isolates recovered from oral rinse samples of healthy individuals without periodontitis. To our knowledge, this type of analysis has not been reported for isolates recovered from periodontitis patients. This study was undertaken in order to determine whether particular clonal groups were associated with periodontitis.

MATERIALS AND METHODS

Study group. Twenty-one patients with untreated periodontitis attending the Dublin Dental University Hospital and meeting specific inclusion criteria (see below) were included in the study (Table 1). Of these patients, 9/21 (42.9%) were male and 12/21 (57.1%) were female. During assessment, patients were provided with a patient information sheet approved by the Ethics Committee of the Faculty of Health Sciences, University of Dublin, Trinity College Dublin, Ireland. A detailed medical and dental history was taken for each patient, followed by an oral examination including a periodontal examination. A dentist experienced in the treatment of periodontitis carried out all clinical examinations between November 2007 and September 2009. The percentages of periodontal pockets with depths greater than 4 mm and 6 mm were recorded, as was the presence of bleeding on probing (BOP) and suppuration on probing (SOP), all performed according to standard procedures, as described previously (21). At a follow-up appointment at least 1 week after the initial appointment, informed consent was obtained from the patients, and microbial sampling for *Candida* species was carried out as described below. The average age of the patients with periodontitis was 42.9 years (range, 27 to 61 years). In total, 4/21 patients with periodontitis were smokers, and 3 patients wore partial dentures (Table 1).

Fifty healthy subjects were also included in the present study. This group consisted of 21 males and 29 females, with an age range of 26 to 73 years and a mean age of 49.7 years. These healthy subjects were attending the Accident and Emergency Department of the Dublin Dental University Hospital and were sampled by oral rinse, as described below.

Inclusion and exclusion criteria. All patients with periodontitis included in the present study met the following inclusion criteria: they were 18 years of age or older, had at least one periodontal site demonstrating a probing depth of greater than 6 mm and BOP and two further sites demonstrating probing depths of greater than 4 mm and BOP, had a minimum of 4 teeth per quadrant, and had given informed consent. Periodontitis patients and healthy subjects were excluded from the study if they met any of the following criteria: pregnancy or lactation, diabetes or asthma, steroid treatment during the last year, or antibiotic or antifungal treatment in the previous 2 months.

Sampling of the oral cavity, periodontal pockets, and gingival sites for *Candida* species. All samplings of the oral cavity, periodontal pockets, and gingival sites were carried out at the Dublin Dental University Hospital. In order to obtain a quantitative estimate of the *Candida* burden in the oral cavity, patients were instructed to rinse their mouths with 10 ml of sterile distilled water provided in a 50-ml plastic sample container for 30 s and then to return the washings to the same container. Rinse samples were transported to the microbiology laboratory for processing within an hour. Upon receipt, a 1-ml aliquot was transferred into a sterile 1.5-ml Eppendorf Safe-Lock microcentrifuge tube (Eppendorf, Hamburg, Germany) and vortexed for 1 min, after which time a 0.1-ml sample was plated in duplicate onto CHROMagar Candida medium (CHROMagar, Paris, France) and incubated at 37°C for 48 h in a static incubator (Gallenkamp, Leicester, United Kingdom).

Subgingival sites selected for sampling included one healthy subgingival site with an absence of BOP and the two deepest periodontitis sites observed during clinical examination that displayed BOP. No healthy sites were available for sampling for two patients with periodontitis (patients 2

TABLE 1 Clinical characteristics of patients with periodontitis[a]

Patient	Age (yr)	Sex	Partial denture wearer	Smoker	% of periodontal pockets with BOP	% of patients with pocket depth of:		Healthy and periodontitis site sampled in patients with periodontitis			
						>4 mm	>6 mm	Site	Probing depth (mm)	BOP	SOP
1	27	M	No	Yes	83.3	55	27.7	Healthy site	2	No	No
								Periodontitis site 1	5	Yes	No
								Periodontitis site 2	5	Yes	No
2[b]	40	F	Yes	No	93	93.5	91	Periodontitis site 1	7	Yes	No
								Periodontitis site 2	7	Yes	No
3	47	F	No	No	13	87	20	Healthy site	2	No	No
								Periodontitis site 1	8	Yes	No
								Periodontitis site 2	7	Yes	No
4	61	M	No	No	45.6	25.3	9.3	Healthy site	2	No	No
								Periodontitis site 1	6	Yes	No
								Periodontitis site 2	6	Yes	No
5	36	M	No	No	69.6	35.1	5.3	Healthy site	2	No	No
								Periodontitis site 1	4	Yes	No
								Periodontitis site 2	6	Yes	No
6	45	F	No	No	61	44	21.5	Healthy site	2	No	No
								Periodontitis site 1	10	Yes	No
								Periodontitis site 2	7	Yes	No
7	35	M	No	No	63	50	20	Healthy site	2	No	No
								Periodontitis site 1	6	Yes	No
								Periodontitis site 2	7	Yes	No
8	55	M	No	Yes	85.8	49.9	15.4	Healthy site	2	No	No
								Periodontitis site 1	6	Yes	No
								Periodontitis site 2	6	Yes	No
9	28	F	No	No	96.5	45.3	2.7	Healthy site	2	No	No
								Periodontitis site 1	7	Yes	No
								Periodontitis site 2	7	Yes	No
10	37	F	No	No	76.5	39.2	5.8	Healthy site	2	No	No
								Periodontitis site 1	5	Yes	No
								Periodontitis site 2	5	Yes	No
11[b]	45	M	Yes	Yes	100	77.7	21.1	Periodontitis site 1	7	Yes	Yes
								Periodontitis site 2	8	Yes	No
12	49	F	No	No	79.8	60.4	25.6	Healthy site	3	No	No
								Periodontitis site 1	9	Yes	No
								Periodontitis site 2	5	Yes	No
13	45	M	No	No	56.8	28.1	12.6	Healthy site	2	No	No
								Periodontitis site 1	10	Yes	Yes
								Periodontitis site 2	5	Yes	No
14	45	M	No	No	10.5	11.5	33.3	Healthy site	3	No	No
								Periodontitis site 1	6	Yes	No
								Periodontitis site 2	6	Yes	No
15	56	F	No	No	39.8	15.2	7.2	Healthy site	2	No	No
								Periodontitis site 1	6	Yes	No
								Periodontitis site 2	10	Yes	No

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TABLE 1 (Continued)

| Patient | Age (yr) | Sex | Partial denture wearer | Smoker | % of periodontal pockets with BOP | % of patients with pocket depth of: | | Healthy and periodontitis site sampled in patients with periodontitis | | | |
						>4 mm	>6 mm	Site	Probing depth (mm)	BOP	SOP
16	36	F	No	No	61.3	8.6	1.3	Healthy site	2	No	No
								Periodontitis site 1	4	Yes	No
								Periodontitis site 2	5	Yes	No
17	31	F	No	No	75	70	4	Healthy site	2	No	No
								Periodontitis site 1	8	Yes	No
								Periodontitis site 2	7	Yes	No
18	36	F	No	No	68.8	25.8	3.2	Healthy site	2	No	No
								Periodontitis site 1	5	Yes	No
								Periodontitis site 2	4	No	No
19	30	M	No	No	87.5	39.5	7.8	Healthy site	2	No	No
								Periodontitis site 1	4	Yes	No
								Periodontitis site 2	6	Yes	No
20	61	F	Yes	Yes	42.5	29.6	2.7	Healthy site	3	No	No
								Periodontitis site 1	4	Yes	No
								Periodontitis site 2	5	Yes	No
21	56	F	No	No	73.1	60.1	21.2	Healthy site	2	No	No
								Periodontitis site 1	7	Yes	No
								Periodontitis site 2	7	Yes	No

[a] Abbreviations: BOP, presence of bleeding on probing of the periodontal pocket; SOP, presence of suppuration on probing of the periodontal pocket; M, male; F, female.
[b] No healthy sites were available for sampling for this patient.

and 11) (Table 1). Sampling sites were isolated, and supragingival plaque was removed with cotton wool pellets. Samples were obtained by using three no. 35 sterile paper points per site (Steri-cell; Coltene Whaledent, Altstätten, Switzerland). The paper points were then immediately placed into a sterile 1.5-ml plastic tube (Sarstedt Ltd., Wexford, Republic of Ireland) containing 1 ml of yeast-peptone-dextrose (YPD) broth (10 g yeast extract per liter [Sigma-Aldrich Ltd., Dublin, Republic of Ireland], 20 g Bacto peptone per liter [Difco, Detroit, MI], and 20 g glucose per liter [pH 5.5]) and sealed. A curette sample of the subgingival plaque deposits from each site was also subsequently taken. A sterile disposable curette (Swede Dental AB, Örebro, Sweden) was inserted into each site, to the depth of the pocket in the case of periodontal sites; applied against the root surface; and moved coronally. Following sampling, the tip of the curette was removed and rotated vigorously in 1 ml of sterile YPD broth in a 1.5-ml tube in order to disperse the microorganisms (26). Subgingival paper point and curette samples were transferred to the microbiology laboratory in YPD transport medium, stored at 4°C, and processed within 24 h.

Prior to culturing, subgingival paper point and curette samples contained in YPD broth were vortexed at maximum speed by using a Heidolph vortex (Heidolph, Schwach, Germany) for 30 s and centrifuged at $2,600 \times g$ for 3 min, and the pellet was resuspended in 1 ml YPD broth by vortexing for 30 s. Following this step, 0.1 ml was plated in duplicate onto CHROMagar Candida agar medium and incubated at 37°C for 48 h in a static incubator. The detection limit of all of these sampling procedures was 10 CFU.

Identification of Candida isolates. Following incubation, CHROMagar Candida plates were examined, and the relative abundance of different-colored colonies was counted and recorded. Selected isolates of each colony color and morphology were purified by subculturing for further analysis. Isolates were initially presumptively identified on the basis of colony color and morphology: *C. albicans* isolates were distinguished from *C. dubliniensis* isolates on the basis of the darker blue-green appearance and smaller colony size of *C. dubliniensis*, in contrast to the larger,

paler green colonies formed by *C. albicans* upon primary isolation in this medium (10). Definitive identification was undertaken by determining *Candida* isolate substrate assimilation profiles using the API ID 32C yeast identification system (bioMérieux, Marcy l'Etoile, France), as described previously (13). Confirmation of the identification of *C. albicans* and *C. dubliniensis* isolates was also carried out by PCR experiments using species-specific *ACT1*-specific primers (4). Several single-colony isolates of each color present on primary CHROMagar Candida plates were identified for each clinical sample. Following identification, isolates were stored on plastic beads in Microbank cryogenic vials (Pro-lab Diagnostics, Cheshire, United Kingdom) at −80°C.

Routine Candida culture. *Candida* isolates were routinely cultured on YPD agar or in YPD broth at 37°C. Liquid cultures were grown overnight at 37°C in an orbital incubator (Gallenkamp) with shaking at 200 rpm.

Chemicals, enzymes, and oligonucleotides. Analytical-grade or molecular biology-grade chemicals were purchased from Sigma-Aldrich or Fisher Scientific Ltd. (Loughborough, United Kingdom). GoTaq DNA polymerase was purchased from the Promega Corporation (Madison, WI).

Nucleic acid isolation. The extraction of DNA from *Candida* isolates for PCRs was performed as described previously by McManus et al. (17).

ABC genotyping of C. albicans. Template DNAs extracted from selected *C. albicans* isolates were assigned to genotype A, B, or C based on the differential PCR amplification of the internal transcribed spacer region of the 25S rRNA gene, as previously described (14).

Genotyping of C. dubliniensis. Template DNA was tested in separate PCR amplification experiments with each of the primer pairs G1F/G1R, G2F/G2R, G3F/G3R, and G4F/G4R to identify the ITS genotype of each *C. dubliniensis* isolate, as described previously (6).

MLST. *C. albicans* isolates selected for multilocus sequence typing (MLST) were recovered from curette, paper point, and oral rinse samples and were chosen, where possible, to represent different periodontal pockets. These isolates were subjected to MLST analysis as previously described

(17). Selected *C. dubliniensis* isolates were subjected to MLST by using an optimized scheme described previously by McManus et al. (16). All DNA sequencing reactions were undertaken commercially by Source Bio Science LifeSciences (Dublin, Republic of Ireland), using an ABI 3730xl DNA analyzer. Sequence analysis was performed by the examination of chromatogram files using ABI Prism Seqscape software, version 2.6 (Applied Biosystems, Foster City, CA).

The identification of genotypes, allelic profiles, and STs was achieved by using the consensus *C. albicans* MLST website (http://calbicans.mlst .net/) or by using a local *C. dubliniensis* MLST database (16, 18). Clade and CC allocations were carried out by using previously identified reference STs (22) with unweighted-pair group method using average linkages (UPGMA) dendrograms and by using eBURST, respectively, as described previously (22). Phylogenetic analyses were carried out by using the MEGA software program, version 5 (12).

For comparative genetic analyses of oral *C. albicans* isolates recovered from patients with periodontitis and those recovered from healthy individuals, *C. albicans* isolates recovered from oral rinse samples of 50 healthy volunteers attending the Accident and Emergency Department at the Dublin Dental University Hospital were also investigated.

Statistical analyses. Fisher's exact tests or Student *t* tests were carried out on data sets in the current study by using GraphPadQuickCalcs (GraphPad Software).

RESULTS

Clinical characteristics of patients with periodontitis. The average percentage of periodontal sites exhibiting BOP was 65.8% ± 24.6%. In total, 45.1% ± 23.7% of periodontal pockets had a depth of >4 mm, and 17.1% ± 19.4% of periodontal pockets had a depth of >6 mm (Table 1).

***Candida* species and cell densities recovered from patients with periodontitis.** *Candida* species were recovered from 10/21 oral rinse samples and/or periodontal sites of 10/21 (47.6%) patients with periodontitis. *C. albicans* isolates were recovered from 10/21 (47.6%) patients with periodontitis, and *C. dubliniensis* isolates were recovered from 5/21 (23.8%) patients with periodontitis (Table 2). All of the *C. dubliniensis* isolates recovered were coisolated with *C. albicans*. *Candida kefyr* was isolated with *C. albicans* from one subgingival sample taken from patient 2. *Candida parapsilosis* was isolated from one subgingival site in patient 11 and was the only yeast species isolated from this site (Table 2); however, a *C. albicans* isolate was also recovered from another site in this patient by paper point. For the 10 patients from whom *Candida* species were isolated from an oral rinse sample, *Candida* species were also isolated from their subgingival sites. Interestingly, for 9 of these 10 patients from whom *Candida* species were recovered by oral rinse, *Candida* species were also recovered at higher densities from periodontal sites in each patient. In two patients (patients 2 and 11), the *Candida* species isolated from the subgingival niche included species not isolated from the oral rinse sample, reflecting site-specific colonization and the differing ecological niche of the subgingival site compared to that of the oropharynx in general. Interestingly, *C. albicans* isolates were recovered from a healthy site in patient 16 by using both curette and paper point sampling; this was the only periodontitis patient in the study from whom *Candida* spp. were recovered from a healthy site (Table 2). *Candida* sp. density counts ranged from 10 CFU/curette sample to 30,000 CFU/curette sample (average, 3,528 ± 8,743 CFU/curette sample), from 10 CFU/paper point sample to 40,000 CFU/paper point sample (average, 3,910 ± 5,466 CFU/paper point sample), and from 10 CFU/ml to 8,000 CFU/ml (average, 1,536 ± 2,384 CFU/ml) for oral rinse samples. The average

Candida density counts recovered from periodontal pockets (3,719 CFU/sample) was 2.4-fold higher than the average *Candida* density counts recovered from oral rinse samples (1,536 CFU/ml) from the same periodontitis patients.

***Candida* species and cell densities recovered from healthy volunteers.** Oral rinse sampling of 50 healthy volunteers was also carried out. This sampling was included in the present study for comparisons of oral *Candida* carriage rates in periodontally healthy individuals with those in periodontitis patients. *Candida* species were recovered from 16/50 (32%) of these patients. *C. albicans* was recovered from all 16 of these individuals, and 1 sample also yielded *C. glabrata*. *Candida* cell densities ranged from 20 CFU/ml to 1,080 CFU/ml (average, 279 ± 317 CFU/ml) for oral rinse samples (individual patient data not shown). Average counts from oral rinse samples of *Candida*-positive healthy carriers (279 CFU/ml) were 5.5-fold lower (*P* = 0.05, determined by a two-tailed unpaired Student *t* test) than average counts from oral rinse samples of periodontitis patients (1,536 CFU/ml).

MLST analysis and ABC genotyping of *C. albicans* isolates from periodontitis patients and healthy oral carriers. Thirty-one *C. albicans* isolates recovered from periodontitis patients were subjected to MLST and yielded 19 distinct sequence types (STs). Two of the MLST alleles (*SYA*-178 and *VPS13*-242) and 13 of the STs recovered from patients with periodontitis have not been identified previously. More than one ST was identified in five of these patients; isolates belonging to at least two different STs were identified in patients 2, 5, 7, 16, and 21 (Table 3). Five isolates recovered from patient 16 using different sampling methods (paper point, oral rinse, and two curette samples) yielded four distinct STs. All five isolates recovered from patient 10 were identified as belonging to ST 444 despite being recovered from different sites and using different sampling methods (Table 3). The majority (21/31) of *C. albicans* isolates recovered from patients with periodontitis belonged to MLST clade 1, the most predominant MLST clade in the *C. albicans* population structure (22). Similarly, 26/31 of the *C. albicans* isolates were found to belong to *C. albicans* ABC genotype A, and 5 were found to belong to genotype B (Table 3). *C. albicans* isolates belonging to MLST clade 1 and ABC genotype A were recovered from 6/9 and 7/9 periodontitis patients, respectively.

The 16 oral *C. albicans* isolates from healthy carriers were also subjected to MLST. Unique STs were identified for each of these 16 isolates: 4 isolates belonged to MLST clade 1, 5 belonged to MLST clade 2, 4 belonged to MLST clade 4, 1 belonged to MLST clade 5, 1 belonged to MLST clade 11, and l belonged to MLST clade 15 (Table 3). The majority of these isolates (13/16) belonged to ABC genotype A, and the remaining 3 isolates belonged to genotype B (Table 3).

Genetic comparison of oral *C. albicans* isolates recovered from periodontitis patients and from healthy individuals. To test the genetic relatedness of the *C. albicans* populations recovered from the two subject groups, a neighbor-joining tree based on *p*-distance was constructed by using the concatenated MLST sequences. Only unique STs were included in the analysis; any duplicate STs were omitted in order to reduce bias. For several patients, the isolates recovered yielded distinct STs, which were very closely related to each other. The concatenated MLST sequences for these STs differed by the loss of heterozygosity in 3 nucleotide sites or fewer, suggesting microvariation in persisting

TABLE 2 *Candida* species and cell densities recovered from patients with periodontitis[a]

	Candida species recovered from sample type						
		Paper point samples (CFU/sample)			Curette samples (CFU/sample)		
Patient	Oral rinse samples (CFU/ml)	Healthy subgingival site	Periodontitis site 1	Periodontitis site 2	Healthy subgingival site	Periodontitis site 1	Periodontitis site 2
1	*C. albicans* (80), *C. dubliniensis* (10)	NCI (0)	*C. albicans* (1,240), *C. dubliniensis* (170)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
2	*C. dubliniensis* (8,000)	No healthy sites available	*C. albicans* (8,000)	*C. albicans* (160)	No healthy sites available	*C. albicans* (30,000), *C. kefyr* (1,200)	*C. albicans* (10)
3	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
4	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
5	*C. albicans* (310), *C. dubliniensis* (80)	NCI (0)	NCI (0)	*C. albicans* (20,000)	NCI (0)	*C. albicans* (10)	*C. albicans* (680)
6	*C. albicans* (80), *C. dubliniensis* (110)	NCI (0)	Not tested	Not tested	NCI (0)	*C. albicans* (1,300)	*C. albicans* (1,000)
7	*C. albicans* (2,660)	Not tested	Not tested	Not tested	NCI (0)	*C. albicans* (280)	*C. albicans* (230)
8	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
9	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
10	*C. albicans* (550)	NCI (0)	*C. albicans* (283)	*C. albicans* (1,090)	NCI (0)	*C. albicans* (1,240)	Not tested
11	*C. albicans* (730)	No healthy sites available	*C. albicans* (10)	*C. parapsilosis* (4,600)	No healthy sites available	NCI (0)	NCI (0)
12	*C. albicans* (1,210)	NCI (0)	*C. albicans* (10)	*C. albicans* (4,520), *C. dubliniensis* (150)	NCI (0)	NCI (0)	*C. albicans* (570), *C. dubliniensis* (570)
13	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
14	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
15	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
16	*C. albicans* (780)	*C. albicans* (40,000)	*C. albicans* (2,680)	NCI (0)	*C. albicans* (20,000)	*C. albicans* (2,970)	*C. albicans* (1,090)
17	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
18	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
19	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
20	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)	NCI (0)
21	*C. albicans* (760)	NCI (0)	*C. albicans* (1,900)	*C. albicans* (6,020)	NCI (0)	*C. albicans* (710)	*C. albicans* (490)

[a] NCI, no *Candida* spp. isolated.

TABLE 3 MLST STs, allelic profiles, and ABC genotypes of oral *C. albicans* isolates recovered from periodontitis patients and healthy carriers[a]

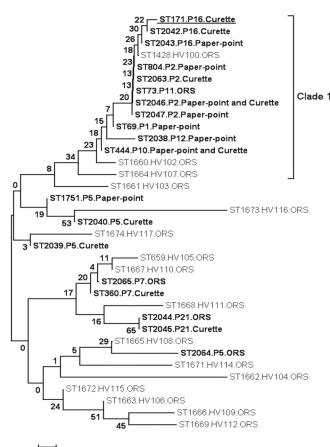| Patient	Isolate	Sample (site)[b]	ST	Allele type							CC	MLST clade	ABC genotype
				AAT	*ACC*	*ADP*	*PMI*	*SYA*	*VPS*	*ZWF*			
Periodontitis													
P1	RM36	PP (1)	69	2	5	5	2	2	6	5	1	1	A
P2	RM3	C (2)	2046	2	3	2	65	2	6	5	1	1	A
P2	RM6	PP (2)	2046	2	3	2	65	2	6	5	1	1	A
P2	RM8	C (1)	2046	2	3	2	65	2	6	5	1	1	A
P2	RM15	PP (1)	2047	2	3	2	31	2	27	5	S	1	A
P2	RM9	C (1)	2063	2	3	2	2	2	6	20	1	1	A
P2	RM10	C (1)	73	2	3	2	2	2	6	5	1	1	A
P2	RM14	PP (3)	804	2	3	2	2	2	20	5	1	1	A
P5	RM1	C (1)	2039	60	7	40	1	7	11	15	12	11	A
P5	RM2	C (1)	2040	60	7	21	1	7	114	15	12	11	A
P5	RM4	PP (1)	1751	60	7	21	1	7	11	15	12	11	A
P5	RM5	PP (1)	1751	60	7	21	1	7	11	15	12	11	A
P5	RM16	ORS	2064	60	7	21	1	6	126	15	S	11	A
P7	RM41	C (1)	360	8	14	8	4	7	3	35	2	4	B
P7	RM43	C (2)	2065	8	7	8	4	7	3	35	2	4	B
P7	RM49	ORS	2065	8	7	8	4	7	3	35	2	4	B
P10	RM28	PP (2)	444	2	5	5	4	2	6	5	1	1	A
P10	RM34	C (1)	444	2	5	5	4	2	6	5	1	1	A
P10	RM27	C (1)	444	2	5	5	4	2	6	5	1	1	A
P10	RM25	PP (2)	444	2	5	5	4	2	6	5	1	1	A
P10	RM29	PP (2)	444	2	5	5	4	2	6	5	1	1	A
P11	RM38	ORS	73	2	3	2	2	2	6	5	1	1	A
P12	RM21	PP (1)	2038	2	2	5	2	2	242	5	1	1	A
P12	RM23	PP (1)	2038	2	2	5	2	2	242	5	1	1	A
P16	RM17	C (1)	2042	8	3	5	2	2	5	5	1	1	A
P16	RM18	PP (1)	2043	8	3	5	2	2	6	5	1	1	A
P16	RM26	C (1)	171	8	3	6	2	2	6	5	1	1	A
P16	RM19	ORS	444	2	5	5	4	2	6	5	1	1	A
P16	RM20	ORS	444	2	5	5	4	2	6	5	1	1	A
P21	RM11	ORS	2044	28	7	38	65	106	122	15	41	8	B
P21	RM12	C (1)	2045	28	7	38	2	106	122	15	41	8	B
Healthy carriage													
HV100	Ca100	ORS	1428	2	23	5	102	2	20	20	S	1	A
HV102	Ca102	ORS	1660	3	5	5	2	2	76	5	1	1	A
HV107	Ca107	ORS	1664	4	5	6	2	2	106	5	S	1	A
HV103	Ca103	ORS	1661	40	24	41	21	4	76	27	S	2	A
HV106	Ca106	ORS	1663	4	5	4	4	139	26	4	2	2	A
HV109	Ca109	ORS	1666	4	2	14	4	139	41	67	S	2	A
HV112	Ca112	ORS	1669	36	2	6	4	49	41	4	S	2	A
HV105	Ca105	ORS	659	11	26	6	4	34	60	119	9	4	B
HV104	Ca104	ORS	1662	3	26	6	4	34	60	55	S	4	B
HV110	Ca110	ORS	1667	8	14	8	4	56	10	8	3	4	A
HV111	Ca111	ORS	1668	14	14	30	4	56	3	8	S	4	B
HV108	Ca108	ORS	1665	5	27	37	4	34	105	12	S	11	A
HV114	Ca114	ORS	1671	4	19	6	4	61	15	201	58	15	A
HV115	Ca115	ORS	1672	4	60	6	4	4	41	4	S	2	A
HV116	Ca116	ORS	1673	2	3	10	2	2	94	2	S	1	A
HV117	Ca117	ORS	1674	13	3	6	34	62	8	47	S	5	A

[a] Abbreviations: HV, healthy volunteer; C, curette; PP, paper point; ORS, oral rinse sample; ST, sequence type; CC, clonal complex.
[b] Sampling methods and periodontal sites from which each isolate was recovered are indicated by the abbreviation and by the site number (in parentheses).

STs in these periodontal pockets. Microvariation of persisting STs was observed for periodontitis patients 2, 5, 7, 16, and 21.

The majority of the STs identified in isolates recovered from patients with periodontitis (21/31) belonged to clade 1; these clade 1 STs were recovered from 6/9 (71.4%) patients from whom recovered *C. albicans* isolates were subjected to MLST. In contrast, only 4/16 (25%) of the STs identified in isolates recovered from 16 healthy *Candida* carriers belonged to this clade (Table 3 and Fig. 1). Surprisingly, one of the STs (ST 1673) recovered from a healthy volunteer separated from the rest of the clade 1 isolates when analyzed by neighbor-joining analysis. In order to investigate this further, a separate neighbor-joining tree containing additional clade 1 STs was constructed (data not shown). In this tree, the outlying ST clus-

FIG 1 Neighbor-joining tree based on the polymorphic nucleotides in 19 of the unique STs identified in 31 *C. albicans* isolates recovered from 9 different patients with periodontitis and 16 unique STs identified from oral rinse samples of 16 periodontally healthy patients. Sequence types identified among isolates recovered from patients with periodontitis are indicated in boldface type, and the patients and sampling methods from which these STs were recovered are indicated alongside each ST. The ST identified for the isolate recovered from the healthy site of periodontitis patient 16 (P16) is indicated by boldface type and underlining. Sequence types identified among isolates recovered from periodontally healthy patients are indicated in normal typeface, and the patients and sampling methods from which these STs were recovered are indicated alongside each ST. The scale indicates *p*-distance. Eleven distinct STs recovered from 6/9 separate periodontitis patients belonged to clade 1, whereas only 4/16 STs recovered from healthy subjects belonged to this clade. Nearest-neighbor analysis of the STs in this tree indicated that the distribution of STs recovered from patients with periodontitis and those recovered from healthy individuals differed significantly ($P = 0.04$, determined by Fisher's two-tailed exact test). Abbreviations: OC, periodontally healthy oral *Candida* carrier; ORS, oral rinse sample.

tered neatly into clade 1, so this outlier is most likely due to the limited number of STs included in the analysis.

Nearest-neighbor analysis of the STs in this tree indicated that the distributions of STs recovered from patients with periodontitis and those recovered from healthy individuals differed significantly ($P = 0.04$, determined by Fisher's two-tailed exact test), as indicated by the differing clade distributions (Table 3 and Fig. 1).

MLST analysis of *C. dubliniensis* isolates recovered from patients with periodontitis. A *C. dubliniensis* isolate recovered from patient 1 and two *C. dubliniensis* isolates recovered from patient 12 were examined by using the *C. dubliniensis*-specific MLST

scheme. One of these isolates was recovered from patient 12 by using curette sampling and was identified as belonging to ST 7, and the other isolate was recovered from this patient by oral rinse and was identified as belonging to ST 50. The third isolate examined was recovered by curette sampling of patient 1 and was also identified as belonging to ST 7. The ITS genotypes of these three isolates were also examined, and the isolates were revealed to belong to ITS genotype 1, the most commonly identified genotype (data not shown). These data showed that these isolates belonged to MLST clade C1, the most densely populated clade in the population structure of *C. dubliniensis*.

DISCUSSION

The purpose of the present study was to investigate the prevalence, cell density, and relative abundance of *Candida* species in periodontal pockets, healthy gingival sites, and the oral cavity of patients with untreated periodontitis. Isolates of the most prevalent species identified, *C. albicans*, were subjected to MLST analysis, and the resulting data were compared with the corresponding data for *C. albicans* isolates recovered from healthy oral carriers of *Candida* without periodontitis in order to determine if there was any association of specific clonal groups with the disease. This type of in-depth population analysis has not been undertaken previously for *C. albicans* isolates recovered from patients with untreated periodontitis.

Although the group of patients with untreated periodontitis in the current study is relatively small ($n = 21$), sampling was undertaken at an average of 3 separate sites per patient and using three different sampling methods, including curettes, paper points, and oral rinse sampling. To date, there have been no investigations comparing paper point and curette sampling techniques in the investigation of the isolation of yeast species from periodontal sites. For this reason, we have used both sampling techniques in the present study. This comprehensive sampling regimen was carried out in order to obtain an accurate representation of the *Candida* species prevalent in the periodontal pockets of patients with periodontitis.

The chromogenic medium CHROMagar Candida was used to culture *Candida* species from clinical samples in order to enhance the detection of separate *Candida* species. Several different *Candida* species commonly isolated from humans exhibit characteristic colony colors on this medium, enabling the detection of mixed species in individual clinical specimens. *Candida* isolates were subsequently definitively identified by substrate assimilation profile analysis and by PCR. Several previous studies that reported *Candida* species isolated from periodontitis patients used conventional mycological media such as Sabouraud's agar for the primary isolation of *Candida* species (5, 27, 33). However, colonies of several *Candida* species can appear to be indistinguishable following primary culture on such media, resulting in a failure to detect mixed species from clinical specimens (2).

Isolates of the two most prevalent species, *C. albicans* and *C. dubliniensis*, were further characterized by ABC or ITS genotyping as well as by MLST. To our knowledge, this is the first study to examine *C. albicans* and *C. dubliniensis* isolates recovered from periodontal pockets by MLST, which is now considered the gold standard for molecular typing and population analysis of *Candida* species. Previous studies of subgingival *Candida* isolates relied on less-reproducible, subjective techniques such as RAPD and electrophoretic karyotyping (1, 24, 33). The advantage of MLST is that

it relies on species-specific DNA-sequence-based comparisons of isolates, and the data can be stored electronically in databases. Data from new isolates can be added gradually to the existing database and can be compared directly with data for isolates recovered from other study groups.

C. albicans was the most commonly identified *Candida* species in this group of patients with periodontitis, which is in agreement with data from several previous studies (1, 5, 8, 10, 32, 35). This species was most commonly recovered from 10/21 (47.6%) periodontitis patients both by oral rinse sampling and by the sampling of periodontal pockets using both paper points and curettes. Similarly, *C. albicans* was the species most frequently isolated from the oral rinse samples of periodontally healthy subjects, being recovered from 16/50 (32%) subjects; only 1 of these healthy patients harbored a non-*C. albicans* species, *C. glabrata*, which was coisolated with *C. albicans*. The average *Candida* density counts recovered from periodontal pockets were 2.4-fold higher than the average *Candida* density counts recovered from oral rinse samples from the same periodontitis patients. Furthermore, average counts from oral rinse samples from *Candida*-positive healthy carriers were 5.5-fold lower than average counts from oral rinse samples from periodontitis patients.

For patients 2 and 11, *Candida* species recovered from periodontal pockets were not recovered by oral rinse sampling of the same patient or by the sampling of an alternative periodontal pocket (Table 2), indicating site-specific colonization. Urzúa et al. previously observed that the oral mucosae of 94 to 100% of patients with periodontitis were colonized with *Candida* species, whereas only 44% of these patients showed *Candida* colonization of periodontal pockets (35). In the present study, the oral mucosae of 9/21 (42.9%) patients with periodontitis were colonized by *Candida*, and 10/21 (47.6%) of these patients showed *Candida* colonization of periodontal pockets, which demonstrates that *Candida* species are not a definitive cause of periodontitis. However, it is likely that *Candida* may exacerbate the condition, especially in periodontal pockets harboring high *Candida* densities. Furthermore, the results of our study showed that individual periodontal sites are specifically colonized by distinct *Candida* species that are not found on the oral mucosa or in other periodontal pockets, suggesting some specific enrichment of particular isolates or species at these sites. These results suggest that oral rinse sampling does not accurately reflect *Candida* populations present in periodontal pockets. Overall, these findings suggest *Candida* proliferation in at least some periodontal pockets and that the *Candida* counts detected were not due simply to contamination by saliva. Nineteen of the 21 periodontitis patients had healthy subgingival sites available for sampling for *Candida*, but only one of these sites yielded *Candida* isolates. The healthy site sampled from patient 16 yielded very high counts of *C. albicans* (20,000 CFU/curette sample and 40,000 CFU/paper point sample); however, only 780 *C. albicans* CFU/ml were recovered in the oral rinse sample from this patient (Table 2). The probing depth (2 mm), the lack of bleeding and suppuration upon probing at this healthy site (Table 1), and the lack of local inflammation suggested that the tooth and the site were healthy. It is difficult to explain this anomalous finding.

MLST analysis of *C. albicans* isolates from periodontitis patients revealed that 13/19 (68.4%) of the STs identified were previously unidentified and that the majority of these isolates belonged to clade 1, the most predominant MLST clade in the population structure of *C. albicans*. These STs were recovered from 6/9 (67.7%) patients for whom recovered *C. albicans* isolates were subject to MLST, suggesting an enrichment of this clade with isolates recovered from periodontitis patients. There did not appear to be a considerable difference in STs identified for isolates recovered from oral rinse samples and periodontal pockets, as for patient 10, separate isolates recovered by rinse, curette, and paper point methods were all identified as belonging to ST 444. Interestingly, two *C. albicans* isolates recovered from patient 16 were also identified as belonging to this ST (Table 3). An examination of the *C. albicans* MLST database (http://calbicans.mlst.net/) revealed that isolates recovered from blood, the esophagus, and the oral cavity of patients in Hong Kong, Brazil, the Netherlands, and Ireland have been identified as belonging to ST 444. The presence of this ST in more than one patient may reflect a high prevalence of this particular ST in the population at large.

Two isolates recovered from patient 21 by curette and oral rinse were identified as belonging to ST 2045 and ST 2044, respectively, but these isolates differed by only one site of heterozygosity in one allele. In contrast to the STs associated with periodontitis, only 4/16 (25%) STs of isolates recovered from the 16 separate healthy subjects belonged to MLST clade 1, whereas the rest of the isolates belonged to other clades (Table 2). Nearest-neighbor analysis and Fisher's exact testing supported the significant difference in the clade distributions between oral *Candida* carriers and patients with periodontitis (Fig. 1). Previous studies reported that *C. albicans* MLST clade 1 is enriched with isolates recovered from superficial infections as well as with ABC genotype A isolates (22). The current study shows that clade 1 is also enriched with isolates recovered from periodontitis patients. Previous to the current study, neither the *C. albicans* nor *C. dubliniensis* MLST databases contained any ST information on isolates recovered from periodontal pockets or on isolates recovered by using curettes or paper point sampling. This information has now been entered into the respective *C. albicans* and *C. dubliniensis* MLST databases for use in future analyses.

Interestingly, *C. dubliniensis* isolates were corecovered with *C. albicans* in 5/21 (23.8%) patients with periodontitis in the current study. The prevalence of *C. dubliniensis* in this group of 21 patients with periodontitis (23.8%) is higher than the 3.5% usually reported for the oral cavities of healthy individuals (25). Indeed, no *C. dubliniensis* isolates were recovered by oral rinse sampling of the 50 periodontally healthy patients included in the present study. Three of the isolates recovered from two of the patients with periodontitis were subjected to MLST, which showed that these isolates were representative of the isolates typically recovered from the human oral cavity; all three of these isolates belonged to ITS genotype 1 and MLST clade C1. This clade is the most heavily populated and least divergent clade, accounting for 66% of the 94 isolates examined by staff at this laboratory to date (16, 18).

This study is the first to investigate *Candida* isolates recovered from periodontitis patients by MLST, which is highly reproducible and reliable in determining the genetic relatedness between isolates of the same species. The current study used three different sampling methods to examine the *Candida* species and densities present in the periodontal pockets and the oral mucosa of periodontitis patients, comparing them with those present the oral mucosa of healthy subjects attending the same hospital. It is possible that the differences in *Candida* densities between periodontal pockets and oral rinse samples and the differential population

distribution between isolates recovered from patients with periodontitis and those recovered from periodontally healthy individuals may reflect some selection of adapted isolates of *Candida* species to this more anaerobic environment or to other pathogens and their products present in the plaque-biofilm associated with periodontitis. It is not possible to say definitively that the presence and/or abundance of *Candida* in the periodontal pockets contributes to the progression of periodontitis or if it is simply a marker for the worsening of the condition. However, given the array of virulence factors, such as secreted aspartyl proteinases, that are expressed by *C. albicans* and the high cell densities often observed at periodontitis sites, it is likely that these organisms contribute significantly to the destruction of periodontal tissues. The novel findings of this study highlight the need for further investigations of this patient group. Such a study should examine a larger number of periodontitis patients on a longitudinal basis and should also involve the application of MLST to recovered *Candida* isolates, analyzing multiple isolates recovered from the each periodontal site and examining multiple periodontal sites.

ACKNOWLEDGMENT

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