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Review

Molecular Techniques and Target Selection for the Identification of *Candida* spp. in Oral Samples

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Abstract: *Candida* species are the causative agent of oral candidiasis, with medical devices being platforms for yeast anchoring and tissue colonization. Identifying the infectious agent involved in candidiasis avoids an empirical prescription of antifungal drugs. The application of high-throughput technologies to the diagnosis of yeast pathogens has clear advantages in sensitivity, accuracy, and speed. Yet, conventional techniques for the identification of *Candida* isolates are still routine in clinical and research settings. Molecular approaches are the focus of intensive research, but conversion into clinic settings requires overcoming important challenges. Several molecular approaches can accurately identify *Candida* spp.: Polymerase Chain Reaction, Microarray, High-Resolution Melting Analysis, Multi-Locus Sequence Typing, Restriction Fragment Length Polymorphism, Loop-mediated Isothermal Amplification, Matrix Assisted Laser Desorption Ionization-mass spectrometry, and Next Generation Sequencing. This review examines the advantages and disadvantages of the current molecular methods used for *Candida* spp. Identification, with a special focus on oral candidiasis. Discussion regarding their application for the diagnosis of oral infections aims to identify the most rapid, affordable, accurate, and easy-to-perform molecular techniques to be used as a point-of-care testing method. Special emphasis is given to the difficulties that health care professionals need to overcome to provide an accurate diagnosis.

Keywords: diagnosis; infection; oral candidiasis; oral health; species identification

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1. Introduction

Candida albicans belongs to our normal mucosal surface's microbiota, from where it may emerge as a pathogen causing local infections, such as inflammation in the oral cavity and *Candida* vaginitis [1,2]. *Candida* species are still the most common cause of fungal diseases worldwide: these yeasts cause infections that range from superficial mucosal membranes to life-threatening invasive diseases, entailing extensive medical or surgical treatment [3–5]. *Candida* spp. exist as commensals, and as opportunist pathogens, being able to compromise various organs and cause diseases in immunocompromised or critically ill patients [6–9]. Therefore, for clinical purposes, the identification of *Candida albicans* per se does not have clinical relevance, especially in colonized environments such as the mouth.

Candida can lead to infection due to changes in the host environment. The infection process is dependent on the host tissue integrity and its ability to maintain normal microbiota, as well as on a healthy immune system. A change in the balance of the resident microbiota, such as the placement of dental implants [10,11], can result in favorable environmental conditions for the proliferation of organisms with the potential for host invasion [12].

Candidemia, a bloodstream infection, is a serious hazard to hospitalized patients, being considered the most clinically relevant form of *Candida* infection [13]. Candidemia is the

most common invasive infection, with mortality rates reported in clinical settings ranging from 30 to 60% [14,15]. The species responsible for the infection has different susceptibility patterns to antifungal drugs, representing a serious challenge for patient treatment. Although oral candidiasis is also an important form of *Candida* infection occurring in the oral cavity, the amount of information available on candidemia is overwhelmingly more abundant.

Despite the clinical relevance of *Candida* spp., the distinction between different species causing oral candidiasis is often difficult. Although there are several molecular techniques currently available for the identification of *Candida* spp., the different strengths and weaknesses associated with these techniques mean it is difficult to reach a consensus on the adoption of an optimal identification method. Any diagnostic tool needs to combine a certain set of attributes in terms of accuracy, specificity, and cost, and at the same time, it must be user-friendly and as non-time-consuming as possible. Therefore, the purpose of this review is to analyze the molecular methods currently used for the detection of *Candida* spp. with a special focus on *Candida* involved in oral infections. A comparative analysis in terms of each method's accuracy, specificity, cost, time, and complexity will be formed. At the same time, we also describe currently used molecular targets as well as others with the potential to improve oral candidiasis diagnosis.

2. Epidemiology of *Candida* Infections

Several Candida species are commensal and colonize the human skin and mucosal surfaces either in a free cell form or in a biofilm. Biofilms, a dynamic community of surface-associated microbes, are protected by an extracellular polymeric matrix and are strongly related to Candida's infection [16–18].

Candida albicans is considered the most common Candida species associated with infection in humans, being often linked to life-threatening situations in elderly, immunocompromised, or critically ill patients [19–21]. The increasing number of invasive surgical procedures, the extensive use of broad-spectrum antimicrobials, and the prevalence of clinical illness, especially in infant and elderly populations, are some of the reasons for the globally increased incidence of candidemia [22]. The Centers for Disease Control and Prevention (CDC) estimate that approximately 25,000 cases of candidemia occur in the United States of America each year [23]. Non-C. albicans species cause approximately two-thirds of candidemia cases in the USA [24], with C. auris being considered a relevant emergent pathogen [15]. In 2019, an epidemiologic meta-analysis was performed in Europe, showing the increasing incidence rate of candidemia with a higher proportion of Candida spp. other than C. albicans [25].

Mortality among patients with invasive candidiasis is as high as 40%, even when receiving antifungal therapy [22,26]. High mortality rates in Candida infections are in part justified by diagnostic inaccuracy (i.e., incorrect identification at the species level and of the drug resistance profile), which may compromise the administration of adequate antifungal therapies and ultimately lead to the patient's death [27–29]. The criteria for initiating Candida antifungal therapy remains poorly specified and often contributes to the widespread prescription of antifungal drugs with no regard for toxicity risks, resistance selection, and unnecessarily high costs of antifungal treatments [30–33].

Oral candidiasis ("thrush") is an opportunist oral mucosa fungal infection [1] that can result in serious health complications and ultimately spread through the bloodstream and lead to candidemia. Furthermore, this infection is commonly associated with elderly patients, frequently leading to longer treatments and higher costs for the health systems. Regarding the diagnosis of oral candidiasis, saliva has been used as a target sample, being already used in the diagnosis of other oral and systemic diseases, such as oral cancer [34,35] and SARS-CoV-2 [36].

The antifungal agents in use for the treatment of oral candidiasis are polyenes (nystatin and amphotericin B), allylamines (terbinafine), and azoles (fluconazole, itraconazole, voriconazole, and ketaconazole). A major concern is the misuse of antimycotic agents

contributing to antifungal resistance in Candida (Table S1). Miranda-Cadena and colleagues characterized Candida oral isolates and showed that most C. glabrata isolates are susceptible to miconazole and nystatin, but resistant to fluconazole and itraconazole. In the same study, Candida parapsilosis isolates were susceptible to fluconazole while azole cross-resistance to miconazole and itraconazole was noted [37]. Increased resistance to antifungal compounds, especially to azoles and to amphotericin B, was already reported [38–41]. All isolates investigated by Anjejo and colleagues (2011) were susceptible to amphotericin B, and 50% of the C. glabrata isolates were resistant to fluconazole [42]. The specific species of Candida responsible for candidemia and Candida spp. that cause oral candidiasis have different susceptibility patterns. Interestingly, Candida krusei susceptibility patterns show multidrug resistance patterns when they are isolated from both oral and blood samples (Table 1).

Table 1. Susceptibility patterns of *Candida* species from blood and oral samples. AmB amphotericin B, FLU fluconazole, ITRA itraconazole, VOR voriconazole, POS posaconazole, MICA micafungin; CAPS caspofungin, S-susceptible, I-intermediate, R-resistant [43–46].

	Blood Samples							**Oral Samples**		
	Azoles				**Echinocandins**			**Azoles**		
	AmB	**FLU**	**ITRA**	**VOR**	**POS**	**MICA**	**CASP**	**AmB**	**FLU**	**VOR**
C. albicans	S	R	S	I	I-R	S-I	S-I	S	S	S
C. tropicalis	S	R	S	S-I	I-R	S	S-I	S	S	S
C. parapsilosis	S	R	S	S-I	S	S	S	-	-	-
C. glabrata	S-I	R	I	S-I	I-R	S-I	S	S	S	S
C. krusei	S	R	S-I-R	S	S	S	S	S	R	S
C. lusitaniae	S	I	S	S	S	S	S	-	-	-
C. auris	S-I	R	R	R	-	R	R	-	-	-

3. Molecular Identification of *Candida* spp.

A definitive diagnosis of candidiasis does not rely merely on its detection in the oral cavity. Since Candida spp. are commensal organisms, a negative culture result, for example, has a greater diagnostic significance than a positive culture result. Conversely, a positive culture result for Candida does not mean that the patient has oral candidiasis. Furthermore, a negative result is only relevant if the techniques used can identify all members of the genus.

Several Polymerase Chain Reaction (PCR) and non-PCR-based methods are used for the molecular identification of Candida spp. The search for a precise, fast, and low-cost identification of fungal species is a great challenge in mycology [47], especially when dealing with species complexes. Phenotypic-based identification is frequently inconclusive.

Techniques based on PCR usually target fungal pathogens by using species–specific probes or primers [47–49]. Conventional PCR, semi-nested and nested PCR, PCR-enzyme immunoassay, various types of real-time PCR, and multiplex PCR have all been used for the in vitro detection of *Candida* species, both qualitative and quantitatively [50]. PCR-based methodologies are often applied in the diagnosis of fungal infections, although they can differ considerably in terms of the outcome. These techniques can be applied for the detection of antifungal resistance-inducing mutations, the quantification of fungal load, and the antifungal therapy surveillance and pathogenesis of *Candida* infection [51]. The PCR-based approaches rely on broad or genus-specific primers that amplify conserved rRNA regions that are sequenced afterwards [52,53] or subjected to other techniques such as analysis of polymorphic sequences (RFLP: Restriction Fragment Length Polymorphism; AFLP: Amplified Fragment Length Polymorphism; RAPD: Random Amplification of Polymorphic DNA; STR: Short Tandem Repeats) [54,55], high-resolution melting analy-

sis (HRMA) [46,54,56,57], microarray-based detection [58,59], and capillary electrophoresis [60]. Capillary electrophoresis is better suited than classical electrophoresis for DNA separation due to its superior speed, efficiency, sensitivity, and simpler suitability for automation [61]. However, the use of this technique creates the need for complex equipment adding complexity to equipment maintenance and specialized personnel.

Existing PCR protocols require enhancements in sensitivity, standardization, and swiftness, as well as a decrease in complexity, in order to be applicable for routine clinical diagnostics [48]. There is a considerable lack of information related to protocols and techniques to identify Candida spp. from oral samples, especially when compared to blood.

3.1. Conventional PCR

Using conventional PCR, Tata and colleagues (2018) were able to identify different *Candida* species from oral samples [62]. *Candida albicans* was the most common (80.9%), followed by *C. tropicalis* (7.2%), and *C. glabrata* (5.3%). The region selected for the amplification was the *ITS2* (Internal Transcribed Spacer 2) of *C. albicans* and *C. dubliniensis* rDNA using fungal-specific primers (Table S1).

Shi (2016) used oral samples from 20 denture-wearing patients (10 with denture stomatitis and 10 healthy denture wearers) and used PCR (targeting *ITS*, Table S1) to assess each denture sample for the presence of *Candida* and other fungi and bacteria. In total, 90% of the samples from the stomatitis group had *Candida* while in only 50% of those from the healthy group was a positive identification found [63].

3.2. Real Time-PCR

Conventional PCR amplifies the DNA target that is later detected with an end-point analysis. In real-time PCR, the amplification product is assessed as the reaction evolves, in real time, which gives RT-PCR the possibility to track the amplification signals in real time. As for pitfalls, RT-PCR has the need for consistency with regard to reagents used [64], and careful consideration in the assay design, template preparation, and analytical methods [65].

Real-time PCR has been widely used for the identification of *Candida* spp. in blood and tissue samples [66], but the same does not apply for oral samples [67]. RT-PCR (using *ITS* as target) was used to identify *Candida* species in patients suffering from oral candidiasis, after piercing the tongue [68], with denture-induced stomatitis [69], and with diabetes mellitus [70].

3.3. Nested PCR

A nested polymerase chain reaction was designed to increase PCR sensitivity by re-amplifying PCR products. Two sets of primers are used in two successive reactions, where the second set intends to amplify a second target within the PCR product from the first run [71]. To limit the amplification of non-specific products, the first reaction allows amplification for a low number of cycles. The second primer set must amplify exclusively the target product from the first amplification and not non-specific products. Nested-PCR was used by Kanbe and colleagues to amplify the DNA topoisomerase II genes of *C. kefyr*, *C. krusei*, *C. tropicalis*, *C. dubliniensis*, *C. parapsilosis*, *C. guilliermondii* and *C. lusitaniae* [72]. The DNA topoisomerase II gene sequence includes highly conserved regions separated by species-specific regions [61]. Kanbe et al. [72] conducted a nested-PCR amplification, in which genomic DNA was amplified with a degenerated primer pair (Table S2), followed by the additional amplification using primer mixtures, to improve specificity.

Nested PCR requires more reagents than conventional PCR, an extra set of primers, and one extra round of agarose gel electrophoresis, becoming a costly and time-consuming method. Additionally, a second amplification reaction increases the risk of sample contamination.

3.4. Multiplex PCR

In Multiplex PCR, several pairs of primers are used to target simultaneously different DNA sequences. This technique takes advantage of the high copy number of rRNA genes,

length, and sequence variability of the *ITS* regions of *Candida* spp. A comparison study between phenotypic methods and multiplex PCR portrayed this last one as a high-accuracy diagnostic tool [73]. Some authors used multiplex PCR to distinguish clinically important *Candida* species from oral samples [74–77] and blood [78]. A diagnostic strategy was created targeting approximately twenty clinically relevant yeast species, *Candida* included. The results were 100% consistent with the MALDI-TOF MS data [79].

Table S1 contains detailed information on primers' sequence and annealing temperature for *Candida* spp. Multiplex PCR provides rapid and effective results. In oral samples, the elimination of the DNA extraction step saves sample preparation time avoiding hazardous or expensive chemicals [80]. Although this method is used in some clinical laboratories, it requires proficiency in primer design and protocol optimization [81].

3.5. Restriction Fragment Length Polymorphism (RFLP)

RFLP uses unique patterns in DNA fragments after enzyme digestion (using restriction enzymes), to genetically differentiate organisms. The distance between these cleavage sites differs between each organism—the resulting restriction fragments—which can be separated by gel electrophoresis arranged by size [82].

RFLP can be used in combination with PCR. Williams and co-workers amplified, by PCR, a region of the *ITS* rRNA gene from 84 *Candida* isolates, including 29 from oral samples. The PCR was designed to amplify intergenic spacer regions of the rDNA with established primers (Table S3) [55]. Isolates of *C. albicans*, *C. tropicalis*, *C. stellatoidea*, *C. parapsilosis*, and *C. krusei* were identified following the restriction digestion of the PCR products.

The PCR-RFLP protocol used by Cirak and colleagues (2003) was successfully applied for the identification of five *Candida* species [83]. The choice of the specific and correct restriction enzyme is a pivotal point. Digestion with the restriction enzyme *Hae*II was effective to differentiate *C. albicans* from non-*Candida albicans*, while *Bfa*I digestion was useful to distinguish *C. parapsilosis* from *C. krusei*. The *Nla*III restriction enzyme was effective in differentiating the *C. parapsilosis* complex [84]. The predicted fragment sizes for different enzymes with the respective species are depicted in Table S4. Other studies were able to discriminate *Candida* species from clinical samples through RFLP, using the D1/D2 region of the 28S rDNA [84], the secondary alcohol dehydrogenase-encoding gene (*SADH*) [85], and the *ITS* region. RFLP analysis is considered a useful, rapid, and trustworthy method [9,86,87].

However, the additional steps of enzyme digestion add further complexity and time in comparison with assays that rely exclusively on PCR-based methods. The time necessary for PCR–RFLP assay can be similar to routine phenotypic conventional methods [87] but it is more sensitive. The storage (refrigeration) and use of restriction enzymes are considered expensive [88], adding to the resulting complex patterns which may be difficult to interpret.

3.6. Microarray

Microarrays consist of thousands of DNA sequences attached to a solid surface. They allow the detection of the presence of genomic DNA regions or the quantification of the expression of genes. The low number of studies using microarrays to identify *Candida* species is notable. The high cost per sample of a single experiment, when compared with sequencing, may be a factor that led to the disuse of microarray for species identification. On the other hand, microarrays depend on specific sequences, and therefore whole genome or RNA sequencing have clear advantages when compared to microarray technologies.

Microarrays can be applied not only to species identification but also to strain typing with high levels of specificity, sensitivity, and throughput capacity. In terms of molecular typing, microarrays were used to identify and obtain different sequence variants of specific DNA sequences. Oligonucleotide probe sequences for the identification of different *Candida* spp. [89,90] are in Table S4.

Microarrays were used in the identification of *Candida* spp. from clinical samples, mostly blood [58,91]. Campa and colleagues used the arrayed-primer extension technique

(APEX) in which the direct labeling of PCR products is not required. This technology combines the advantages of Sanger dideoxy sequencing with the high-throughput potential of microarrays [92]. The experiment led to the correct species identification, including of the highly related *C. parapsilosis* complex. The microarray was tested for its specificity with reference strains and blind clinical isolates [58].

The major advantages of gene chip technology are its miniature size, high performance, and process automation. The process of optimization is long to ensure stable, specific, sensitive, and reproducible results. The discrimination between specific and unspecific signals may be a challenge in a mixture analysis, as is the case, for instance, of cross-hybridization [93].

3.7. High-Resolution Melting Analysis (HRMA)

HRMA is a simple, rapid, and inexpensive tool useful in the identification of a broad range of clinically relevant *Candida* species. It is combined with RT-PCR, providing an alternative for directly analyzing genetic variations [94]. Alnuaimi and colleagues (2014) used HRMA using the *ITS* region of rDNA to classify relevant *Candida* spp. from oral samples [46]. The authors identified all species in their list and four different genotypes of *C. albicans* [46]. Another author suggests real-time PCR followed by HRMA directly in the biological samples as an efficient method that takes only 6 h to result [54]. HRMA followed by RT-PCR was more rapid and efficient than the classic biochemical methods used in the study [95].

HRMA has some advantages over other genotyping methods owing to the inexpensive single-step procedure, reducing the risk of contamination when compared to a multistep procedure (such as RFLP or nested PCR). Despite this, the technique does not distinguish between some *Candida* spp. due to Tm (Melting Temperature) ranges overlapping [54].

3.8. Multilocus Sequence Typing (MLST)

MLST is a sequencing-based method that analyses nucleotide polymorphisms in fragments from essential genes, the "housekeeping genes" [96,97]. MLST generates a molecular characterization with high discriminatory power and reproducibility. MLST can be used in the epidemiological differentiation of several clinical isolates from *Candida* species and polymorphism search [98–100]. MLST has been used to obtain information about allele diversity in *C. tropicalis* [101] and to access the evolution of virulence-associated mechanisms of the emergent pathogen *C. krusei* [102].

3.9. Loop-Mediated Isothermal Amplification (LAMP)

Amongst all of the currently available isothermal amplification techniques, only Nucleic Acid Sequence-based Amplification (NASBA) [103], Rolling Circle Amplification (RCA) [104], Transcription Mediated Amplification (TMA) [105], and LAMP have been used in the identification of *Candida* spp. Nonetheless, to the best of our knowledge, LAMP is the only technique that has been applied to oral samples.

LAMP is an isothermal one-step amplification method that uses two inner primers (FIP: Forward Inner Primers and BIP: Backward Inner Primer), and two loop primers creating a continuous loop structure during DNA amplification. LAMP uses a Bst DNA polymerase with increased activity, which can produce a high molecular weight DNA fragment within a short time. LAMP's exceptional specificity is due to a set of four primers with six binding sites that must hybridize correctly to the target sequence before DNA biosynthesis occurs. The detection methods include real-time turbidity, fluorescence probes, and others [106]. The use of LAMP to identify relevant fungi and yeasts has been reviewed by Niessen and colleagues [107]. LAMP has shown very good results in the identification of *Candida* spp. in clinical samples [108], dairy products [109], and oral samples [110]. When using oral samples, LAMP was executed by Noguchi and colleagues but only for the detection of *C. albicans* and not for non-*C. albicans* species.

The key elements for a good LAMP assay are primer design and concentration. A higher concentration of the loop primers, FIP and BIP, provides a faster amplification and therefore a quicker result.

Monitoring LAMP amplification can be performed with a water bath/heating block instead of an (expensive) thermocycler. It is real-time, fast, and has a higher amplification efficiency and sensitivity. Naked eye visual amplification monitoring is possible through the turbidity of magnesium pyrophosphate, a by-product of the reaction, color changes by fluorescent intercalating dyes using a UV lamp, and agarose gel analysis revealing patterns that are characterized by a ladder pattern [111]. A list of primers used for the identification of *Candida* spp. is available in Supplementary material—Table S5.

3.10. Next Generation Sequencing (NGS)

Next-generation sequencing, including Whole-Genome sequencing, can also be used for the identification of *Candida* species [112]. NGS can detect markers of antifungal drug resistance from pathogenic *Candida* strains [113,114], *ITS* variabilities in prevalent pathogenic *Candida* spp. [115] and provide insightful metagenomic studies [116]. NGS provided valuable input in the diagnosis of rare infections such as *Candida* meningitis [117] and pseudomembranous oral candidiasis [118].

Although discontinued, pyrosequencing was the first of the NGS technologies to be commercially available and has provided large amounts of sequence data [119], becoming a technology of historical interest. DNA pyrosequencing, or sequencing by synthesis, became possible in the late 1990s as a rapid, cost-effective alternative to Sanger (di-deoxy) DNA sequencing [120]. Third-generation sequencing, also known as next next-generation sequencing, refers to those technologies that do not depend on the PCR amplification of DNA.

The identification of yeasts, including *Candida* spp., has been performed by pyrosequencing using different targets like 18S rRNA gene [52], *ITS1,* and *ITS2* [63,121,122], with results consistent with classic biochemical tests [121,123,124]. Pyrosequencing has also been used for the identification of *Candida* pathogens in various clinical samples such as vaginal [125], blood [126], and oral samples [127]. Pyrosequencing is only able to read short-length sequences of nucleotides, providing a disadvantage for the technique when the target has a longer sequencer. Pyrosequencing data analysis can be complex and challenging. This approach has provided evidence about mutations, with no known previous association with phenotypic drug resistance of the *ERG* and *FKS* genes in *Candida* spp. [128].

The use of pyrosequencing has declined because of the rise of new methodologies of NGS such as Illumina [112], which are less expensive, provide longer sequences, higher sensitivity to detect low-frequency variants, have a faster turnaround time for high sample volumes, and a comprehensive genomic coverage [129].

3.11. Peptide Nucleic Acid Fluorescence In Situ Hybridization (PNA FISH)

Peptide Nucleic Acid molecules are synthetic DNA fragments in which the negatively charged sugar–phosphate backbone of DNA is replaced with a noncharged polyamide [130]. This grants probes to hybridize to their complementary DNA targets with higher affinity and specificity, which means this technique is perfect for targeting highly secondary structured rDNA molecules [131,132]. The technique has the capacity to identify *Candida* spp. within 2.5 h [133]. PNA confers very low background noise, showing it to have a high sensitivity [71].

The PNA FISH probe test was developed to evaluate multiple *Candida* spp. from blood cultures [134–136]. It encompasses three coverage colors: green, red, and yellow for *C. albicans* or *C. parapsilosis*, *C. glabrata* or *C. krusei*, and *C. tropicalis,* respectively [137]. An alternative multi-*Candida* probe was used by Reller and co-workers to identify all *Candida* species under study [138].

The PNA-FISH assay major throwback is that visualization implies the use of a fluorescence microscope, adding costs to the laboratory equipment. This method has proven to be expensive [139], reaching high economic costs per patient [140].

3.12. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS is a molecular method broadly implemented in modern clinical microbiology laboratories [141]. This approach is a rapid and reliable alternative for yeast identification and consists of the generation of protein 'fingerprints' that are compared with reference spectra [142–144]. MALDI-TOF involves sample ionization with a laser striking a matrix of molecules to cause the analyte molecules to enter into a gas phase without fragmentation. It is coupled with the principle of Time-of-Flight analysis, in which ions of different mass/charge ratios are dispersed in time during their flight along a path of known length (the equipment analyzer) [145].

MALDI-TOF MS has been used for the speedy identification of *C. albicans* and non-*albicans* species on blood [146,147] and oral samples [148–150], with shorter turn-around times and higher accuracy compared to conventional biochemical methods [151]. MALDI-TOF MS was performed on a library composed of clinical and reference strains with an accuracy of 94% when compared with *ITS* sequence analysis [142,152]. This method provided results of genus-specific proteins within 24 h of *Candida* causing bloodstream infections [50,143,153].

MALDI-TOF is a promising technique, but the lack of spectra characterization for microorganisms still needs to be addressed. Without available reference spectra, results cannot be achieved. This availability of spectra seems to be changing through the emergence of new studies, building information about *Candida* spp. [147,154–156]. The equipment cost is one of the strongest disadvantages of a clinical and routine setting.

Figure 1 provides a qualitative comparison of the molecular techniques used for the identification of *Candida* spp. Table 2 presents advantages and disadvantages for each molecular technique.

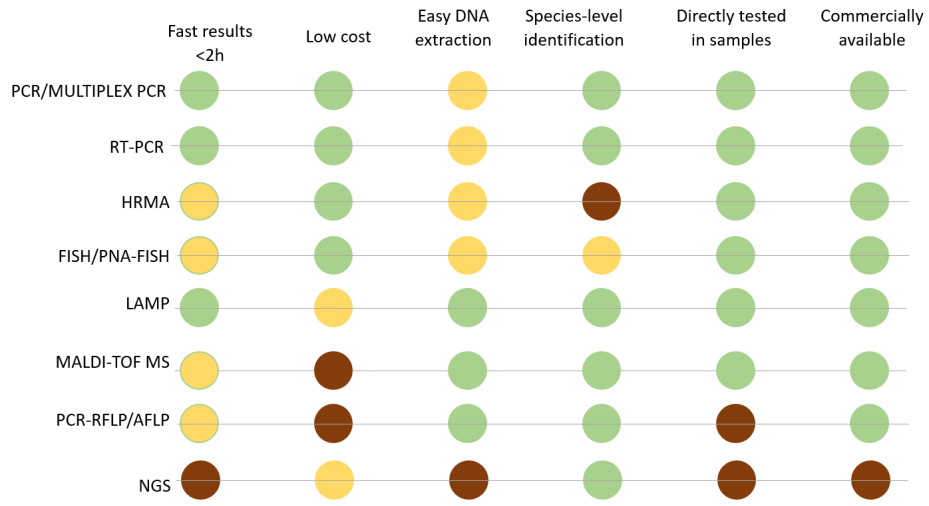

Figure 1. Qualitative analysis of the main molecular approaches for *Candida* species detection. RT-PCR: Real-Time - Polymerase Chain Reaction; NGS: Next Generation Sequencing; HRMA: High-Resolution Melting Analysis; FISH/PNA-FISH: Fluorescent In Situ Hybridization/Peptide Nucleic Acids-FISH; PCR-RFLP/AFLP: Polymerase Chain Reaction - Restriction Fragment Length Amplification/Amplified Fragment Length Polymorphism; LAMP: Loop-Mediated isothermal amplification; PCR-ESI-MS: Electrospray Ionization Mass Spectrometry coupled with broad-spectrum PCR; MALDI-TOF MS: Matrix-Assisted Laser Desorption IonizationTime of Flight Mass Spectrometry; [95,157]. Brown: Low; Yellow: Medium; Green: Good.

Table 2. Resume of advantages and disadvantages for currently used molecular techniques in the identification of *Candida* spp.

Molecular Technique	Advantages	Disadvantages
Conventional PCR	Low cost compared with other PCR-based techniques, low in complexity	Requires an additional amplification detection step
RT-PCR	Real-time detection and quantification, no additional step of detection	Expensive equipment
Nested PCR	Sequence primers available for different gene targets	Requires more reagents than other PCR-based techniques and an additional set of primers. Prone to contamination
Multiplex PCR	Detection of multiple gene targets	Requires an additional amplification detection step
RFLP	High specificity, Sequence primers available for different gene targets	High-cost enzymes and storage, requires an additional amplification detection step.
HRMA	Low risk of contamination when compared to RFLP or nested PCR	Not capable of distinguishing between some *Candida* spp.
MLST	High discriminatory power, useful for epidemiological studies, and evolution of virulence-associated mechanisms	High cost
LAMP	High specificity, high sensitivity, high-speed, low-cost equipment. Several methods for amplification detection	Requires attention in optimization and primer design
NGS	High discriminatory power, large dataset allows for additional analysis	High cost, complex results which require specialized analysis
PNA FISH	Rapid identification of *Candida* spp. in blood cultures	Results visualization adds a cost to equipment
MALDI-TOF MS	High specificity, rapid identification	High cost of equipment, lack of spectra characterization for comparison

3.13. Promising Molecular Techniques: ddPCR

Droplet digital PCR (ddPCR) is a new technology based on water–oil emulsion droplets which provides accurate DNA quantification [158]. ddPCR shows a higher quantitative range in comparison to qPCR for the identification of clinical *Candida* spp. in blood samples, providing an early diagnosis as well as a prognostic value for candidemia [159]. ddPCR has yet to be used with oral samples.

4. DNA Target Selection for the Identification of *Candida* spp.

The selection of a suitable molecular target in the diagnosis of any infectious disease is of pivotal importance. Regardless of the molecular technique, an accurate diagnostic strongly depends on the molecular target specificity and its discriminatory capacity. *ITS* has been selected as the barcode of choice for the identification of fungal species [160]. The same happens for *Candida* spp., with *ITS1* and *ITS2* being widely used. Nonetheless, there are some alternatives available. The *MP65* gene plays a role in maintaining cell wall integrity, adherence to epithelia, and biofilm formation in *C. albicans* [57,161]. DNA

topoisomerase II coding gene is used due to its highly conserved regions, separated by species-specific regions [72].

Other molecular targets related to virulence, pathogenesis, and antifungal resistance can also be useful as a complement in the identification of *Candida* spp. The ergosterol biosynthetic genes, *ERG3*, *ERG5*, *ERG6*, and *ERG11*, are common targets for the detection of antifungal drug resistance to amphotericin B (AMB) [17,162]. A study also showed that potential mutations in the *ERG5* gene confer resistance against AMB [163].

Several genes are useful for MLST, although their use is not widespread. Sequencing can contribute to improving and simplifying current MLST strategies, as recently described for *C. glabrata* and *C. albicans* [164,165]. Additionally, as mentioned in the NGS Section 3.10, sequencing is relevant for metagenomics studies, the identification of drug resistance, and the diagnosis of rare infections. This is crucial for difficult-to-identify or emerging pathogens, such as *C. auris* [166], or for adequate therapeutic directions in drug-resistant species [161].

5. Conclusions

The oral mycobiome is intricate, dynamic and involves extensive biofilm formation. *Candida* is frequently found in the human mouth and, as with several other pathogenic fungi, appears to be an oral resident in some individuals. Because of the similarity between species, the correct identification is difficult but crucial to the success of the therapy outcome. When choosing a technique for *Candida* identification in clinic settings, material costs, the use of trained professionals, the complexity of the technique, the specificity of the results, and time should be taken into consideration.

We focused on a broad range of molecular techniques that have been used for the identification of *Candida* species in oral samples, having in mind that a timely and accurate diagnosis of *Candida* infection is indispensable for timely intervention with appropriate antifungal therapy. To overcome this challenge, a fast, reliable, inexpensive, and uncomplicated point-of-care diagnosis is needed. For *Candida* spp. identification, only a few techniques fit this criterion: from the necessary sample treatment to time-to-result, only LAMP and Multiplex PCR seem to look promising.

LAMP is considered by many authors to be a highly useful diagnostic technique, especially in areas where access to complex healthcare facilities is limited. However, the amount of data and results on the efficiency of this technique when applied to the diagnosis of *Candida* infections are still scarce when compared to other techniques. Furthermore, the clinical application of LAMP on a larger scale has yet to be achieved. LAMP requires the least amount of time out of all the techniques to reach a diagnosis and does not require costly equipment. Using the *ITS* sequence as a target, it is possible to design primers to identify *Candida* spp. sampled from the area of infection. Taken together, LAMP specificities and requirements seem to be the most adequate for the simplest and most time-efficient diagnostic of oral candidiasis. It is worth highlighting that the exponential growth of gene sequence databases has provided the ideal conditions to develop more efficient and reliable primer designs, enhancing target specificity and the accuracy of diagnosis.

Ultimately, the pros and cons of each molecular technique detailed in this review can hopefully help dentists who deal with patients with inflammatory conditions to choose the most appropriate diagnostic method. Nevertheless, novel developments within this field of research may lead to improvements in currently available techniques and to the development of new ones.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12189204/s1, Table S1: Primers (Universal and species-specific) used for *Candida* species detection and size of fragments separated by agarose gel electrophoresis; Table S2: List of primers used for Nested PCR; Table S3: Restriction Fragment Length Polymorphism (RFLP) fragment patterns in oral *Candida* spp. isolates with restriction enzymes; Table S4: Oligonucleotide probes used for *Candida* spp. in non-oral isolates; Table S5: Primers used in Kasahara (2014) for the detection of *Candida* spp. with LAMP.

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