Review

Candida diagnostic techniques: a review

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Received: 16 January 2023 / Accepted: 27 April 2023
Published online: 10 May 2023

Abstract
Fungal infections (mycoses) represent a major health issue in humans. They have emerged as a global concern for medical professionals by causing high morbidity and mortality. Fungal infections approximately impact one billion individuals per annum and account for 1.6 million deaths. The diagnosis of *Candida* infections is a challenging task. Laboratory-based *Candida* species identification techniques (molecular, commercial, and conventional) have been reviewed and summarized. This review aims to discuss the mycoses history, taxonomy, pathogenicity, and virulence characteristics.

Keywords *Candida* · *Candida albicans* · Candidiasis · Diagnosis · Genome · Infection · Mycoses · Yeasts

1 Introduction

Mycoses (fungal infections) are characterized by high morbidity and mortality, which affect a huge global population every year [1–5]. Fungal infections have been a constant threat to humans [6]. The occurrence of fungal diseases is on the rise and hospital-residing immunocompromised patients are particularly the primary victims [7]. The main fungal pathogens (50–90% mycoses isolates) involved in the infections include *Trichosporon* spp., *Aspergillus* spp., *Scedosporium* spp., *Zygomyces* spp., *Paracoccidioides* spp., *Cryptococcus* spp., *Rhodotorula* spp., *Candida* spp., *Histoplasma capsulatum*, *Geotrichum* spp., *Coccidioide simmitis*, and *Fusarium* spp. [8–10]. The yeasts belonging to the *Candida* genus have been frequently isolated from fungal infections [11]. There are almost 200 known *Candida* species whereas almost twenty species are associated with human infections [12]. *Candida*-related human infections are considered a major issue, especially in hospitalized patients suffering from severe underlying diseases and immunodeficient ICU patients [13, 14].

The parasitic fungus, *Candida* species, is a eukaryotic yeast that accounts for about 8% of global nosocomial infections [5, 15]. Taxonomically, *Candida* species belong to the Kingdom Fungi (Mycota), phylum Ascomycota, subphylum Saccharomycotina, class Saccharomycetes, and order Saccharomycetales [16]. The emergence of *Candida albicans* as a common pathogenic yeast has been reported [5, 17–19]. *Candida* clinical isolates that could colonize the human tissues to exert infections include *Candida krusei*, *Candida dubliniensis*, *Candida lusitaniae*, *Candida parapsilosis*, *Candida utilis*, *Candida tropicalis*, *Candida famata*, *Candida glabrata*, *Candida rugosa, Candida kefyr* (pseudotropicalis), *Candida guilliermondii*, *Candida lipolytica*, and *Candida haemulonii* [8, 20–23]. The significant role of non-*Candida albicans Candida* species (NCAC) in invasive candidiasis has also been established [24]. The occurrence of candidiasis is quite higher than the overgrowth of *Candida*. This review discusses mycoses-related topics and describes virulence factors related to candidiasis along with their participation in pathogenicity and future approaches for better candidiasis diagnosis.

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J.Umm Al-Qura Univ. Appll. Sci. (2023) 9:360–377 | https://doi.org/10.1007/s43994-023-00049-2

2 The virulence and pathogenicity of *Candida* spp.

The survivability of *Candida* species under harsh conditions makes them highly detrimental pathogens, which could threaten the lives of immunocompromised patients [25, 26]. Previously, yeast microorganisms were supposed to infect only immunocompromised patients, but virulence factors have revealed their pathogenicity to other patients as well. Multiple aggression mechanisms of these microorganisms participate in the pathophysiology of the disease [27, 28]. Several virulence factors of *Candida* enhance their pathogenicity, which includes invasion and adhesion to inanimate surfaces and body tissues, metabolic adaptation, dimorphism, phenotypic switching, secretion of hydrolytic enzymes, and formation of biofilms [29].

Candida albicans, a polymorphic yeast, exhibits different cellular morphologies such as hyphae, yeast cells (white phase), GUT (commensalism-related), pseudohyphae, chlamydospores, and opaque (mating cell types). Different polymorphic forms affect the pathogenicity of *C. albicans* [30, 31]. The parameters such as nutrients, pH, temperature (37–40 °C), Co_2 concentrations (5.5%), and amino acids facilitate their morphological transition, which is crucial for pathogenicity. The yeast forms could conveniently spread inside the host tissues whereas filamentous shapes possessing higher adhesion capability help in the invasion of the host tissues [32].

Candida spp initially attach to the host cell through adhesion proteins present on the fungal cell surface (*pga1*, *als1-7*, *hwp1*, *als9*, and *eap1*), and immobilized ligands (cadherins, integrins, or other microorganisms). Fungal cells invade the tissue after the adhesion. The invasion and damage of the epithelium are considered pathogenic [8, 33–35]. It could occur via two mechanisms (active penetration or endocytosis) depending upon the type of host cell. For example, the invasion of oral cells by *C. albicans* occurs through active penetration and endocytosis, whereas only active penetration is possible in intestinal invasion [36, 37].

Candida is highly adaptable to various environmental conditions (low oxygen, limited nutrition, pH fluctuations, and nitrosative, cationic, temperature, osmotic pressure, and oxidative stresses) [8, 31, 38, 39]. This adaptability is of key importance for the *C. albicans* pathogenicity [8]. The formation of mycotic biofilm is a complicated process, which generates a highly organized structure (Fig. 1). Multiple studies have been performed to investigate the biofilm formation in *Candida* species [40–43]. The National Institute of Health has reported that more than 80% of the total microbial infections in the United States are caused by fungal biofilms [5]. Biofilm consists of adhered, attached, and accumulated microorganisms that form extracellular polymeric substances (EPSs) to provide a structural matrix [44]. Planktonic *C. albicans* cell adhesion to the surfaces is the initial step that induces an organized strong extracellular matrix (ECM) structure [44]. There are four consecutive *C. albicans* biofilm phases including the Adhesion phase, Initiation phase (early phase), Maturation

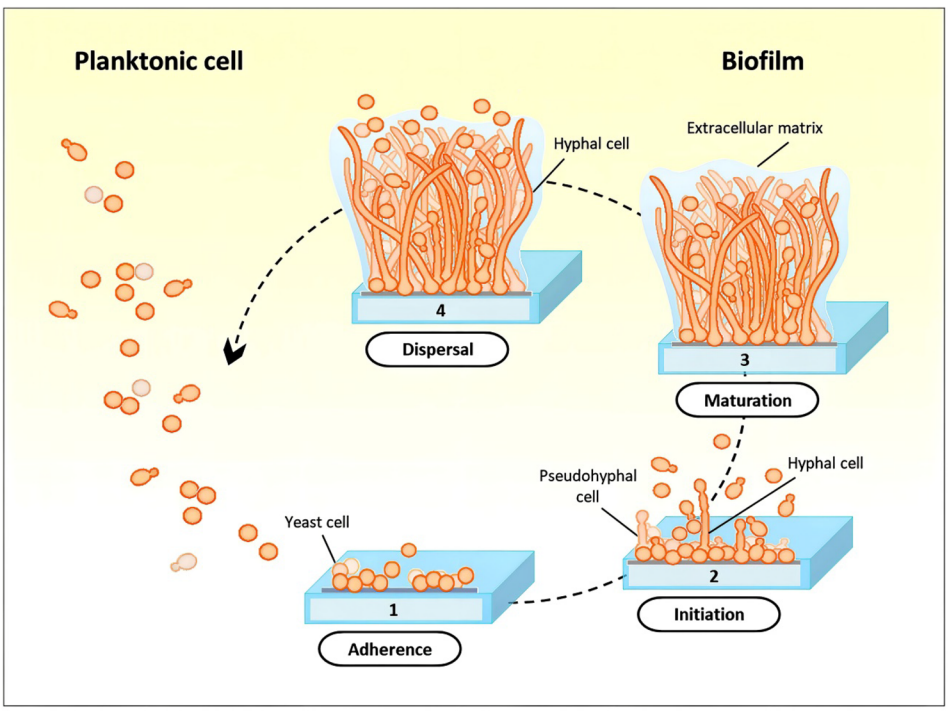

Fig. 1 The consecutive phases of biofilm formation in *C. albicans* (1). Adhesion phase, yeast cells adhere promptly to the surface. (2). Initiation phase (early phase), spherical yeast cells replicate and start to secrete extracellular matrix (ECM) and develop Pseudohyphae. (3). Maturation phase (intermediate phase), the mature biofilm develops with hyphal filaments extending far from a basal layer (yeast forms). (4). Dispersal (dispersion phase), yeast cells disperse from the biofilm and diffuse, expanding the infection and starting the cycle again

phase (intermediate phase), and Dispersal phase (dispersion phase) (Fig. 1).

Extracellular hydrolytic enzymes (phospholipases and proteases) are necessary for the pathogenicity-causing *Candida* yeast virulence factors. These enzymes facilitate the *C. albicans* invasion through host protein degradation (hemoglobin and keratin) and cell membrane structure alteration. These steps help in the targeting and invasion of the host's immunity cells by avoiding antimicrobial agents. Different *Candida* species follow this process including *C. albicans, C. tropicalis, C. parapsilosis*, and *C. dubliniensis* [8].

3 Roles of virulence-associated genes in pathogenicity

Yeasts can induce infection and overwhelm the host defense systems due to the existence of various proteins and genes associated with their pathogenicity, known as virulence factors [45]. *C. albicans* genes encode several pathogenic virulence factors. These genes and their products contribute to mycological pathogenicity and are called virulence factors. Numerous genes associated with *C. albicans* pathogenicity have been described [8, 46, 47]. These factors include the ability of *C. albicans* to transition from yeast form to hyphal form, adhesins, biofilm formation, and hydrolytic enzymes secreted (aspartyl proteases, and phospholipases) [8, 48].

Candida albicans can grow in the form of yeast and mold. The transition between yeast and hyphal forms is termed (dimorphism) [49]. The dimorphism of *C. albicans* is a unique characteristic of yeast pathogenicity. Both morphologies have their role to support their virulence [8]. It has been reported that the form of hyphal is more invasive than the form of yeast [50].

The dimorphism of *C. albicans* plays an important role in the pathogenicity of both systemic and superficial infections. It should be noted that both yeast and filamentous forms of *C. albicans* were detected in infected tissues [51]. The capability of *C. albicans* to switch from yeast to filamentous form contributes to the various nature of its infection phases, such as adherence to epithelial and endothelial cells, invasion, iron acquisition from host sources, biofilm formation, escape from phagocytes, and immune evasion [51].

Adhesins are the yeast surface molecules that intermediate the binding of *C. albicans* to the surface of human or microorganism cells, inert polymers, or proteins [52]. *ALA1, ALS1, Hwp1, INT1, MMT1, PMT1, PMT6,* and, *Als1p* are candidate genes considered as encoding adhesins [52–55]. Other putative adhesins are mannan, chitin, factor 6 oligomannosaccharide, 66-kDa fimbrial protein, fibronectin-binding protein, iC3b binding protein, fucose binding protein, GlcNAc or glucosamine, and secreted aspartyl proteinase (SAP) [52, 53]. The Sapproteins of *C. albicans* were encoded by a family of 10 SAP genes i.e. *SAP1*, *SAP2*, *SAP3*, *SAP4*, *SAP5*, *SAP6*, *SAP7*, *SAP8*, *SAP9*, and *SAP10*. The major functions of the *C. albicans* Saps are nutrition for the yeast cells, assisting penetration and invasion, and avoiding host immune responses [56].

Phospholipases hydrolyze glycerophospholipids, which are the main components of mammalian cell membranes. It destabilizes the membranes by cleaving fatty acids from phospholipids [57]. There are seven phospholipase genes have been identified i.e. *PLA*, *PLB1*, *PLB2*, *PLC1*, *PLC2*, *PLC3,* and, *PLD1*. However, the role of the enzymes encoded by these genes are not yet clear [58]. In a comparative study conducted by Ibrahim et al.[59], evidence was obtained that phospholipase acts as a virulence factor, and a series of *C. albicans* obtained from candidemia patients were compared with the isolates obtained from the oral cavities of healthy people. In candidemia cases, higher phospholipase activity was found, reflecting the virulence of these isolates.

4 Antimycotic-resistance of *Candida*

Candida resistance to various antimycotic agents poses a serious public health concern. The *Candida* incidence in the bloodstream has increased from 2.2 to 3.2 cases/100,000 population/annum in Europe [60, 61]. Antifungal resistance (azoles and echinocandins) in *Candida* could hinder their treatment. *C. parapsilosis* and *C. glabrata* are commonly found clinical strains, which cause invasive candidiasis by modifying prevalence at various locations [62]. Multidrug-resistant (MDR) cases featuring non-*albicans Candida* (NAC) and *C. albicans* strains have raised serious concerns [63]. Antifungal drug preservation has increased because of *Candida auris* based global nosocomial outbreaks featuring higher morbidity and mortality. Centers for disease control and prevention (CDC) added these strains to priority antibiotic resistance threats in 2019 [62]. The world health organization (WHO) convened the first meeting in 2020 for establishing a health-related pathogens (mycoses) priority list. They also defined the research and development (R&D) priorities for encouraging the development of new drugs, diagnosis methods, and strategies. Global antimicrobial-resistance surveillance system (GLASS) of antimycotic resistance (AMR) has developed a protocol to counter *Candida* spp. based bloodstream infections (BSIs). Antimycotic susceptibility data related to blood *Candida* isolates especially from patients in high-risk hospital units (ICUs) is available through GLASS reports [64, 65].

5 Candidiasis

Candidiasis is a global *Candida* yeast-based major human fungal disease. Candidiasis refers to disseminated, visceral, and mucosal-cutaneous infections of the genus *Candida* [66]. These infections could occur at any age and are easily identifiable infection risk factors [67]. Candidiasis infections are complex and of different types. The types are distinguished through different morphology, and relationships between mucosal and immune systems [68]. The epidemiological records have established the association of five species with candidiasis including *C. albicans* (65.3%), *C. glabrata* (11.3%), *C. tropicalis* (7.2%), *C. parapsilosis* (6.0%), and *C. krusei* (2.4%) (Table 1 and Fig. 2) [69].

The infections such as oral candidiasis, vaginitis, candidemia, systemic infections, and cutaneous candidiasis are linked to *Candida*. Different candidiasis types are presented in Fig. 3 [70–72]. Oropharyngeal candidiasis (OPC), also known as "thrush" (different oral mucosal sites and tongue infection) is characterized by the overgrowth of

Table 1 *Candida* species implicated in human infections. Adapted from Dabas, [71]

Most frequent species	Less frequent species	Rare species
C. albicans	*C. dubliniensis*	*C. blankii*
C. glabrata	*C. famata*	*C. bracarensis*
C. tropicalis	*C. inconspicua*	*C. catenulate*
C. parapsilosis	*C. lipolytica*	*C. chiropterorum*
C. krusei	*C. metapsilosis*	*C. ciferri*
C. guilliermondii	*C. norvegensis*	*C. eremophila*
C. lusitaniae	*C. orthopsilosis*	*C. fabianii*
C. kefyr	*C. pelliculosa*	*C. fermentati*
	C. rugosa	*C. freyschussii*
	C. zeylanoides	*C. haemulonii*
		C. intermedia
		C. lambica
		C. magnolia
		C. membranaefaciens
		C. nivariensis
		C. palmioleophila
		C. pararugosa
		C.pseudohaemulonii
		C. pseudorugosa
		C. pintolopesii
		C. pulcherrima
		C. thermophile
		C. utilis
		C. valida
		C. viswanathii

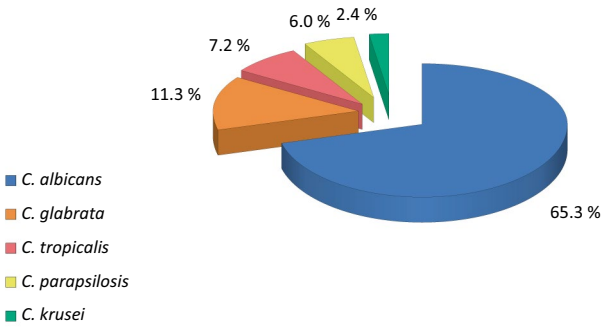

Fig. 2 The most common *Candida* pathogenic species

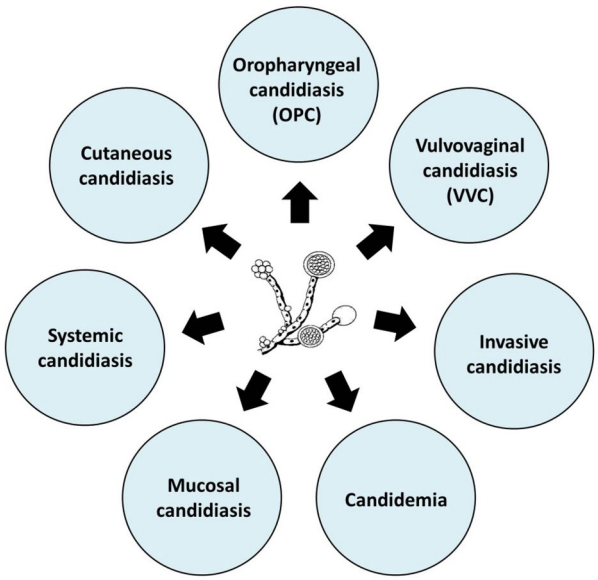

Fig. 3 Major types of candidiasis

mycoses and superficial tissues' invasion [73–75]. Urogenital or Vulvovaginal candidiasis (VVC) is a common mycosis worldwide that infects female genital tracts [76]. This infection is quite frequent in diabetic individuals, pregnant women, and patients under antibiotic and corticosteroid treatments [77, 78].

Invasive candidiasis (IC) can substantially implicate any organ and refers to deep-seated infections such as osteomyelitis (bone infection), peritonitis (tissue covering the inner abdominal wall and organs), and intra-abdominal abscess [71, 79–82]. Candidemia (BSI) refers to the *Candida* species infection in the blood of patients suffering from fever [83]. *Candida parapsilosis* is the most frequent agent of bloodstream infection (BSIs) among non-*C. albicans Candida* species [84]. BSIs-associated *Candida* ranks fourth in nosocomial-associated infections in the USA

whereas it is at the sixth number in Europe [67, 85]. The occurrence of mucosal candidiasis is common and they are more invasive than fungal candidiasis. Candidiasis-infected mucosal surfaces could be oral mucosa, pharynx, esophagus, urogenital, intestine, and urinary system [86, 87]. Systemic candidiasis is also referred to as acute organ invasive or systemic hematogenous candidiasis. During systemic candidiasis, the *Candida* cells spread to the whole body and rapidly form abscesses in vital organs. The infections of systemic candidiasis lead to visceral lesions (deep candidal focus) or *Candida* septicemia (candidemia) [15, 88]. Nail and cutaneous candidiasis is a secondary subacute or chronic infection of predisposed patients, which could be local or spread to the nails and skin. There are various types of cutaneous candidiasis such as otomycosis, intertrigo candidiasis, diaper rash, *Candida* folliculitis, paronychia, and onychia [71].

Candidiasis could also infect the surfaces of different medical devices including urinary catheters, central venous catheters, and cardiovascular devices. These infections are commonly associated with morbidity and deaths of hospitalized patients [89]. *Candida*-related infection of medical devices is among the major pathogenesis-related factors [89–92].

6 Microbiological and clinical tests for the diagnosis of *Candida*

The diagnosis of *candida* infections is complicated. This section summarizes the clinical laboratory tests (molecular, conventional, and commercial) for the isolation and identification of *Candida* species.

6.1 Phenotypic methods

6.1.1 Conventional methods

Non-*albicans* and *C. albicans* identification was initially carried out through phenotypic traits (biotyping, morphotyping, chemical resistance, and serotyping). However, the reproducibility and differentiation levels of these approaches are very low, which limits their reliable diagnosis and epidemiological analysis [73, 93].

6.1.2 Germ-tube test (GTT)

The germ-tube test (GTT) method refers to the formation of tube-like structures by *Candida's* reaction and it primarily identifies and differentiates *C. dubliniensis* and *C. albicans* from other species. This is a simple, rapid, and economical identification technique with 98% sensitivity. *Candida* is incubated in serum at 37 °C for 2–4 h and then observed for the presence of tube structures. This is an effective method that is widely applied for *C. albicans* identification [94, 95].

6.1.3 Chlamydospore formation

The chlamydospore formation test is based on the appearance of chlamydospores during the last stage. This test differentiates *C. albicans* from *C. dubliniensis*. During the test, *C. albicans* is subjected to dormant growth under a controlled environment using agar media (cornmeal and rice extract), which leads to chlamydospore formation [96].

6.1.4 Carbon assimilation

This is an economical, simple but time-consuming test that specifically identifies *Candida* species. The method involves *Candida* growth on carbon substrates followed by incubation and growth examination [97].

6.1.5 Carbohydrates fermentation

Fermentation tests rely on the acid and carbon dioxide formation in the liquid media. Carbohydrates facilitate the fermentation process during the test. However, this *Candida* identification test is complex, time-consuming, and less sensitive as compared to the carbohydrate assimilation test. Traditionally, carbohydrate fermentation test was performed for *Candida* species differentiation, but now this method is not often used [98].

6.2 Rapid identification systems

The commercial and rapid identification methods are based on conventional methods (sugar assimilation, germ tube, and chlamydospore formation tests). However, these methods are comparatively easy, rapid, and cheaper [99].

6.2.1 API 20C Aux system

API 20C Aux (bioMerieux Vitek, Hazelwood, MO, USA) system was developed from API 20 system, which is a

carbohydrates assimilation-based process. It involves the assimilation tests of 19 carbohydrates in cupules that are incubated (24–72 h) at 30 °C followed by turbidity analysis using computer programs [99]. This method accurately differentiates between *C albicans, C. dubliniensis,* and other *Candida* species [100]. However, certain limitations of this method have also been reported during *C. krusei* identification [101].

6.2.2 API Candida system

API *Candida* system containing ten tubes could detect five carbohydrates through various acidification and enzymatic tests (seven). The testing procedures are based on sugars acidification and enzyme activity [102, 103]. This is a highly accurate and simple method that completes without the involvement of computers. It produces observable color changes, and morphological characteristics to identify *Candida* species [103].

6.3 Chromogenic media-based commercial systems

6.3.1 CHROMagar technique

CHROMagar is a rapid, precise, and straightforward *Candida* identification technique. It involves the media with multiple chromogenic substrates, which react with *Candida* species enzymes leading to the growth of colonies in different colors [104, 105].

6.3.2 Candida ID system

Candida ID system (bioMerieux, Marcy l'Etoile, France) is a rapid and more developed CHRMagar system. A chromogenic indolyl glucosaminide substrate in the growth medium reacts with *Candida* species to produce different insoluble colors. Turquoise/ blue color is produced by *C. albicans, C. guilliermondii*, and *C. lusitaniae* whereas *C. tropicalis* produces pink color [106].

6.3.3 Fluorogenic membrane filtration method

This is another developed and accurate CHROMagar method. The enzymatic reaction is detected by passing fluorogenic substrates through a nylon membrane filter. Different *Candida* species (*C. albicans, C. krusei*, *C. tropicalis*, and *C. glabrata*) could be differentiated through this method [107].

6.3.4 Fungichrom I and fungi-fast I twin systems

The fungichrom I twin system (International Microbio, Parcd'activites-allee D'athenes, France) consists of sixteen whereas the fungi-fast twin system consists of ten test cupules. The samples are incubated at 30 °C for 24–48 h and observed for color changes. *Candida* identification rate is high in the fungichrom twin system and it is a more rapid and simple method as compared to the fungi-fast I twin system [108].

6.3.5 Biggy agar system

Biggy agar (Oxoid Company, Wade Road, Basingstoke, Hampshire, UK) is a bismuth sulfite-containing chromogenic medium. *Candida* species convert it to bismuth sulfide. The reaction generates specific colors depending upon the growth of *Candida* species. The color of *C. albicans* becomes light brown whereas the color of *C. tropicalis* changes to dark brown. However, this method could not differentiate among certain species such as *C. krusei* and *C. parapsilosis,* which produce similar colors in their colonies [109].

6.4 Automated methods

Recent automated methods are fast, reliable, and broad, which could facilitate the development of new patient management and therapeutic techniques.

6.4.1 Vitek YBC system

The Vitek YBC system (bioMerieux Vitek, Inc., Hazelwood, MO, USA) is an auto-microbial system that is widely applied in research centers and laboratories. This method could simultaneously perform twenty-six biochemical tests from the same inoculum. Therefore, it could identify several *Candida* species including *C. parapsilosis, C. albicans, C. glabrata,* and *C. tropicalis*. Furthermore, it contains a computerized assessment system for more reliable information on *Candida* species. The handling of this method is easy and does not require an experienced person [110].

6.4.2 VITEK® 2 ID-YST system

VITEK® 2 Yeast identification (YST) is a developmental system. This is a high-speed, rapid, and simple system that could simultaneously conduct multiple reactions. This system can perform 47 carbohydrate assimilation-based fluorescent biochemical reactions including deamination and oxidation reactions with various arylamidases and oxidases of *Candida* species. This system is more suitable for differentiating *C. dubliniensis* and *C. glabrata* in addition to the identification of yeast microorganisms [111].

7 Serological methods

The serological commercial tests are used for the identification of *Candida* identification. There are several highly sensitive reagents for fungal identification. (1-3)-Beta-D-Glucan is a novel reagent to identify fungal infection. Similarly, a new fungal surrogate marker is highly sensitive and specific for the *Candida* infection diagnosis. This test also helps in the diagnosis of Candidemia [112]. A polysaccharide (Galactomannan) of the fungal cell wall could diagnose fungal infection and invasive fungi processes including Candidemia. This laboratory test can track the fungi infection and the patient's state to assess the treatment efficacy [113].

8 Molecular methods

Non-DNA and DNA-based molecular identification methods are gaining popularity. These are highly specific, accurate, and sensitive techniques for the identification and differentiation of *C. albicans* from other *Candida* species. These features encourage their widespread applications [93, 114].

8.1 Non-DNA-based techniques

8.1.1 Multi-locus enzyme electrophoresis (MLEE)

MLEE could estimate enzymatic protein and its polymorphism through gel-based analysis of enzyme migration. The charge and size of the protein molecule determine the length of enzymatic migration. MLEE is used in epidemiology and genetic studies as it could accurately differentiate between unknown and novel strains. However, indirect genome evaluation by this technique is the main disadvantage, which results in slow rates of evaluating variations accumulated in the species and incorrect outcomes. MLEE is also does not detect whole nucleotide variations (Table 2) [115, 116].

8.2 DNA-based methods

DNA-based tests could detect and differentiate among microorganisms' DNA. This category consists of DNA-based conventional methods and Exact DNA-based methods [104].

8.2.1 Conventional DNA-based methods

These are DNA components-based oldest microorganism identification methods. DNA of eukaryotic organisms is extracted through the cell membrane hydrolysis by hydrolyzer enzymes [104].

8.2.2 Pulsed-field gel electrophoresis (PFGE)

Several studies have discussed the application of DNA genome components for microorganism identification, which is derived after the hydrolysis of the cell membrane [117, 118]. Therefore, different electrophoresis methods were developed including pulsed-field gel electrophoresis (PFGE), orthogonal-field alternative gel electrophoresis (OFAGE), contour-clamped homogeneous electric field (CHEF), transverse alternate gel electrophoresis (TAFE), and field inversion gel electrophoresis (FIGE). This is an ideal technique for separating of chromosome-sized DNA molecules due to the size of *C. albicans* chromosomes are about (1- 4 Mb) (Table 2) [117, 118].

8.2.3 Restriction enzyme analysis (REA)

REA technique was initially applied to conduct an epidemiological investigation of *C. albicans* infections. This is a complex method that may require a computer-assisted software. REA involves the purification of the total genomic DNA followed by cleavage through multiple endonuclease enzymes (e.g., EcoRI, MspI, BglII, HinF1, or HindIII) that produces small fragments resulting in a

Table 2 Resume of advantages and limitations of the different rapid approaches for detection of *Candida* spp

Diagnostic approaches	Advantages	Limitation	References
Pulsed-field gel electrophoresis (PFGE)	Separation of chromosome-sized DNA molecules about (1–4 Mb)	Time-consuming	[117–119]
Restriction enzyme analysis (REA)	Rapid identification Straightforward Cost-effective	Difficult to interpret and differentiate strains	[120]
Random amplified polymorphic DNA (RAPD)	Quick Simple and cost-effective High specificity Widely used for the identification and differentiation of *Candida* spp	Poor reproducibility of fingerprints	[121, 122]
Amplified fragment length polymorphism (AFLP)	More reproducible than (RAPD) Reproducible (as a genotyping method)	High cost Rarely used for *C. albicans* typing Complex (multiple-step) Requires high expertise	[122, 123]
Nested PCR	Sequence primers available for several gene targets	Prone to contamination Requires an additional set of primers and reagents more than other PCR-based approaches	[126]
Real-time PCR (RT-PCR)	Accurate Rapid RT-PCR detection and quantification No additional step of detection	Expensive equipment	[125, 126]
Nucleic acid sequence-based amplification (NASBA)	Very sensitive No need for thermal cycling devices	High cost	[128]
Peptide nucleic acid-fluorescent in situ hybridization (PNA-FISH)	Simple approach Rapid identification of *Candida* spp in blood cultures	Results visualization adds a high cost to equipment	[126, 129]
Microsatellite length polymorphism (MLP) typing	Rapid identification High throughput Amenable to automation Recommended for epidemiological studies associated *C. albicans*	MLP lacks a public database and requires additional standardization before achieving this	[130, 131]
Multi-locus sequence typing (MLST)	Useful for epidemiological studies High discriminatory power Evolution of virulence-associated mechanisms	High cost	[126]
DNA-microarrays	Sensitive Rapid Highly specific	Identifies only sequences that the array was supposed to detect Non-coding RNA's that are not yet predicted as expressed are typically not represented on an array	[133, 134]
Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)	Rapid Reliable Widely available Cost-effective	High cost Lacks of spectra characterization for comparison	[126]

sequence-dependent restriction fragment length polymorphism (RFLP). The produced fragments were separated using the agarose gel electrophoresis technique. Then visualized on the gel after dyeing with ethidium bromide. In this technique, the interpretation and differentiation of strains are very difficult due to the production of complex patterns with enormous bands of unequal intensities (Table 2) [120].

8.2.4 Random amplified polymorphic DNA (RAPD)

RAPD is a genomic DNA amplification-based technique. The amplified products are isolated through agarose gel electrophoresis according to the size of the amplified fragments. However, short genomic DNA could generate a complex pattern and segments that are different from the source. RAPD technique is widely performed for the identification and differentiation of *Candida* species, especially *C. albicans* (Table 2) [121, 122].

8.2.5 Amplified fragment length polymorphism (AFLP)

This method involves the hydrolysis enzymes-based digestion of genomic DNA, which is then amplified to obtain different DNA fragments. The fragments are further isolated using a high-performance instrument such as fluorescent dye-labeled primers. The amplification is carried out under highly specific conditions as compared to the RAPD method. Therefore, AFLP is more reliable, accurate, and reproducible than RAPD. However, the application of AFLP for *Candida* spices, especially *C. albicans* is limited as it is comparatively costly and requires an experienced person to perform its multiple complex steps (Table 2) [123, 124].

8.3 Exact DNA-based methods

8.3.1 Polymerase chain reaction (PCR) based-Candida detection methods

PCR is a landmark DNA molecular microbiology technique, which facilitates microorganism identification in human cells. PCR can detect *Candida* species during the invasive fungal infection phase such as Candidemia. PCR could also diagnose various genetic disorders. PCR is a rapid method, which can amplify several DNA fragments within minutes to detect millions of scarce DNA copies [125]. Fungal identification through PCR could be conducted in two ways: (i) Nested PCR technique amplifies DNA molecules in two steps to reduce the amplification errors. Therefore, it is considered a highly accurate method (ii) Real-time PCR could quantify amplified DNA in real-time at each PCR cycle. Real-time PCR is carried out using two types of fluorescence such as labeling probes and recently developed double-stranded dyes. Several studies have recommended the application of real-time PCR as it is more accurate and rapid than nested PCR [125]. PCR amplification (nested PCR or real-time PCR) is followed by the analysis of amplicons and conclusions. Different methods are used for the amplicon analysis, but direct sequencing is the most accurate technique as compared to single-strand conformational polymorphism (SSCP) and polyacrylamide gel electrophoresis (Table 2) [127].

8.3.2 Nucleic acid sequence-based amplification (NASBA)

This technique is based on RNA amplification and does not need a thermal cycling instrument for the specific detection of microorganisms. In contrast to DNA, RNA is quickly degradable outside the microorganism cells. NASBA method uses three expensive enzymes (RNase H polymerase, reverse transcriptase, and T7 RNA polymerase). Therefore, it is widely used for the amplification of *Candida* species. The results are generated within a few hours and it can differentiate up to six different *Candida* species (Table 2) [128].

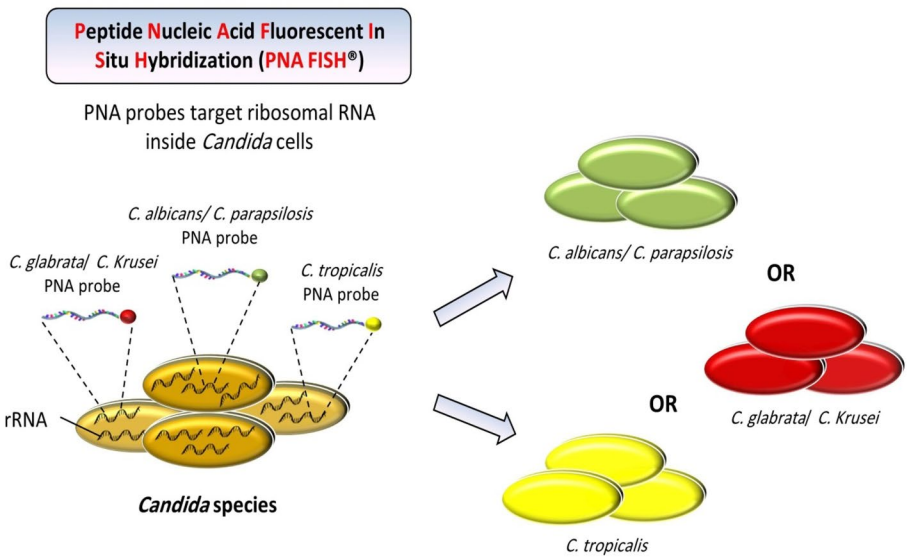

Fig. 4 A schematic drawing of the labeling process with the peptide nucleic acid fluorescence in situ hybridization (PNA FISH®) probe

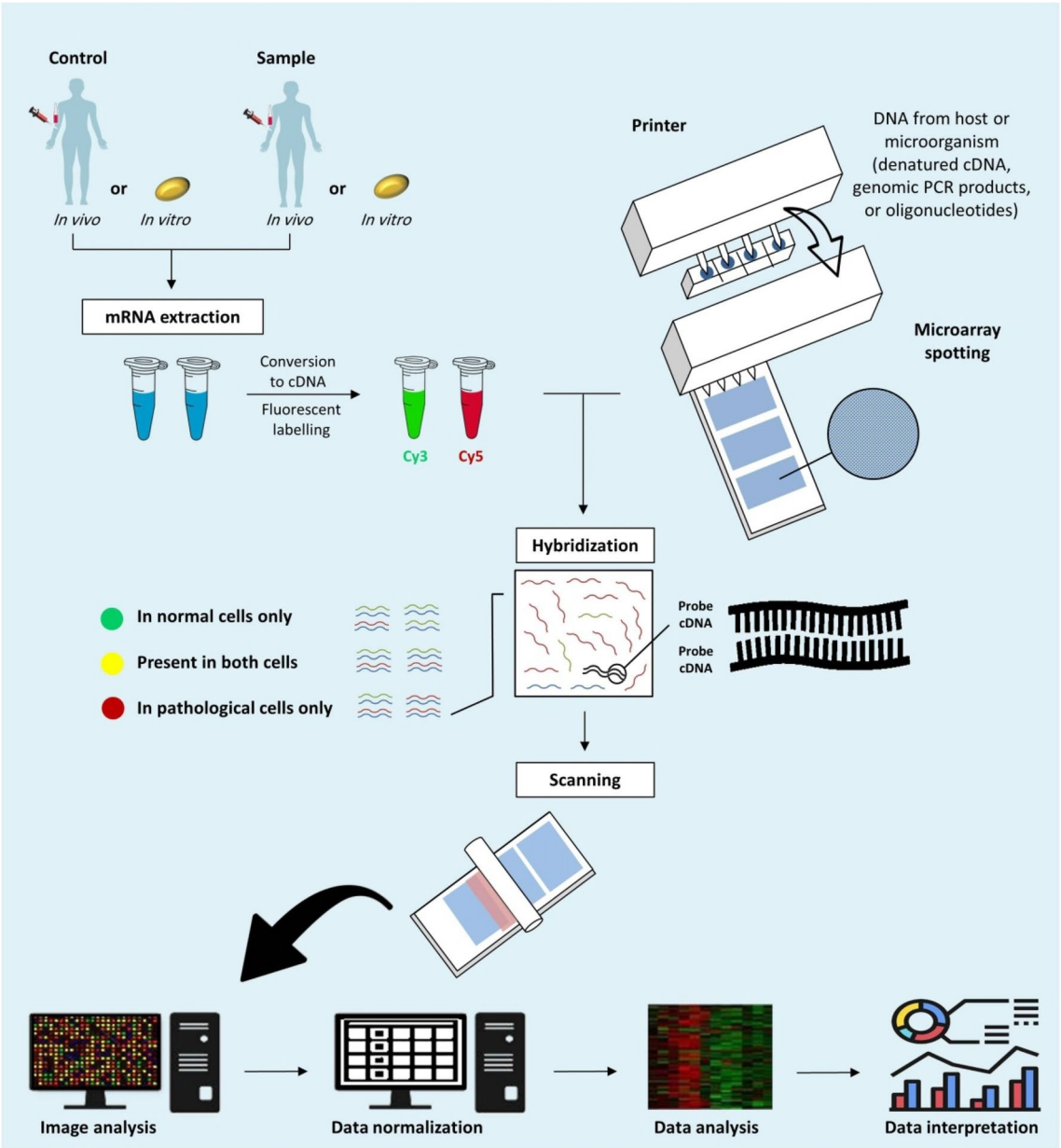

Fig. 5 Overview of the steps involved in DNA-microarray technique

8.3.3 Peptide nucleic acid-fluorescent in situ hybridization (PNA-FISH)

FISH is a classical yeast detection method that does not require purification and isolation steps. It only uses fluorescein-labeled oligonucleotide probes. The accuracy of this method further increases in combination with peptide nucleic acid (PAN) probes. These probes lead to hybridized microorganism cells in the samples, which are examined through advanced fluorescence microscopy. The studies have elaborated that the results of the PAN-FISH technique could be compared to PCR, but it generates the results faster than the PCR technique. Furthermore, the PAN-FISH approach contains highly specific probes for the *Candida* species. Therefore, it could specifically differentiate between *Candida* species (Table 2 and Fig. 4) [129].

8.3.4 Microsatellite length polymorphism (MLP) typing

This technique has been used in a *Candida* species-related epidemiology study. This is a simple, rapid, and highly reproducible method. MLP technique is based on the amplification of microsatellite stretch in the cell nucleotides. The sensitivity of this method depends on the type of microsatellite marker, which detects primer pair flanking in a specific microsatellite area. High polymorphism of amplified microsatellite fragments favors the use of the MLP typing technique in genetic analysis to determine the type of alleles (heterozygous or homozygous). Finally, high-resolution gel electrophoresis measures the allele length. The numerical results could easily be compared for the identification of various microorganisms (Table 2) [130].

8.3.5 Multi-locus sequence typing (MLST)

MLST is based on the amplification of internal fragments of nucleotide sequence polymorphisms in the independent loci genes. These are housekeeping genes, which are

Table 3 Genome differentiation in *Candida* species

Candida species*	Genomic size (mb)	GC content (%)	No. of genes	Average of gene size (bp)	Intergenic average (bp)	Ploidy	Pathogen†
C. albicans WO-1	14.4	33.5	6,159	1,444	921	D	+ +
C. albicans SC5314	14.3	33.5	6,107	1,468	858	D	+ +
C. tropicalis	14.5	33.1	6,258	1,454	902	D	+ +
C. parapsilosis	13.1	38.7	5,733	1,533	758	D	+ +
L. elongisporus	15.4	37.0	5,8.2	1,530	1,174	D	–
C. guilliermondii	10.6	43.8	5,920	1,402	426	H	+
C. lusitaniae	12.1	44.5	5,941	1,382	770	H	+
D. hansenii	12.2	36.3	6,318	1,382	550	H	–

mb megabases, *bp* base pair, *D* Diploid, *H* Haploid

†Relative level of pathogen strength: + +: strong pathogen, +: moderate pathogen, –: rare pathogen

**C. albicans SC5314* assembly 21 and gene set (dated 28 January 2008) downloaded from the *Candida* genome database, *D. hansenii* assembly from GenBank Dujon et al. [137]. The remaining assemblies are reported as part of this work and are available in GenBank and at the Broad Institute *Candida* database website

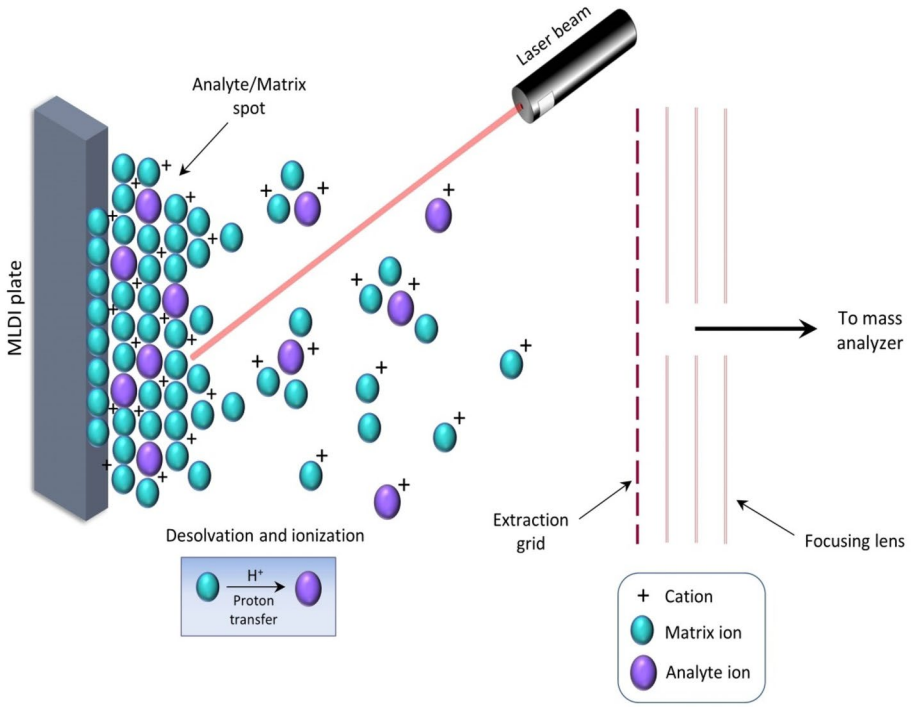

Fig. 6 Diagram showing the MALDI Time of Flight Mass Spectrometry (MALDI-TOF MS) process

selected due to their stability. This technique could amplify the DNA fragments up to the size of 400–500 bp. The difference in alleles corresponding to multi-locus sequence type distinct alleles characterize each housekeeping locus. The ability to analyze only seven sequences in 300–400 bp loci genes is the main limitation of this method. Furthermore, the analysis of diploid microorganisms could generate two identical products in this method whereas they vary in heterozygous bases on the polymorphic databases (Table 2) [132].

8.3.6 DNA-microarrays

Microarrays are microscopic high-density oligonucleotide probes, which hybridize the nucleic acid samples followed by immobilization on a solid surface. Microarray-based systems depend upon strain typing. These are highly sensitive, specific, and automatic standards that do not require a prior database. Microarrays hybridization-based bound sequence is detected using a highly efficient fluorescent scanning instrument and advanced computer software (Table 2 and Fig. 5) [133].

9 Modern methods

9.1 Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS is a modern approach that is now widely available in clinical microbiology laboratories. MALDI-TOF MS is a rapid, cost-effective, reliable, and powerful identification method. MALDI-TOF MS generates protein fingerprints of each microorganism in the sample, which could be easily compared at the reference library. Briefly, the fungi degradation is carried out at the temperature of the curie pyrolysis point followed by the production of volatile fragments from the cleavage of tiny molecules. Finally, the mass spectrums of volatile fragments are analyzed using a mass spectrometer, which represents the fingerprint image of each microorganism. The lack of spectra characterization for comparison is a major limitation of the current analysis (Table 3 and Fig. 6) [126, 135, 136].

9.2 Candida species genomes: genome sequence and comparative identification

A significant variation in composition and size has been reported between the sequenced genomes of *Candida* (Table 3) [137, 138]. Table 3 presents a high continuity range between scaffolds (9–27). Scaffold size and number closely relate to each *candida* microorganism. The field gel electrophoresis could assess the genomes in each *Candida* microorganism where telomeric arrays finally link to the scaffolds. 10.6–15.5 Mb difference could occur in the genome size of approximately 50% of *Candida* species whereas the difference in GC content (guanine and cytosine) could range between 33 and 45% (Table 3). The transportation and repetitive capability of these elements could differ between assemblies in numbers and type [139]. *Candida* species are primarily different in genomic size and phenotype, however, they are quite similar in protein-coding gene numbers (5.733–6.318) as presented in Table 3. The genome of the smallest *Candida* species (*C. guilliermondii*) contains more genes than the genome of the largest *Candida* species (*L. elongisporus*). Therefore, genome size and gene numbers are not correlated [138].

10 Conclusion and promising future directions

The Nanopore sequencing technology is based on the DNA translocation across a lipid-bilayer membrane through a pore, which is formed by *Staphylococcus aureus* alpha-hemolysin after applying the electrical fields [140]. This novel method has been used for mycosis detection in various studies. Ashikawa et al. [141] applied a nanopore sequencing system for the identification of five *Candida* species in positive blood-culture vials and their performance was compared with Sanger sequencing. This system provides rapid optimization of reagents and instruments. This system could further help to develop accurate and rapid point-of-care devices for clinical and field usage [142].

CRISPR-Cas9 (clustered regulatory interspersed short palindromic sequences-CRISPR associated protein 9) versatility has led to the development of an identification tool known as Specific Highly Sensitive Enzymatic Reporter UnLOCKING (SHERLOCK). SHERLOCK could successfully identify target nucleic acids in attomolar concentrations to distinguish closely-related viruses and genotypes up to the difference of a single base pair [143]. Furthermore, Next-generation (SHERLOCK version 2) is a quantitative multiplex analysis that could visualize the final results using lateral flow devices (LFDs) system [144]. SHERLOCK v2 could achieve high specificity and sensitivity in combination with the HUDSON method and rapid DNA extraction techniques. This combination could be successfully used for the diagnosis and

identification of Dengue and Zika viruses. In short, the SHERLOCK v2 system coupled with an efficient DNA extraction tool provides a reliable portable platform to identify mycotic pathogens [145].

This review updated the current overview of *Candida* infections. Other studies have reported an alarming rise in *Candida* disease. This suggests that the current diagnostic methods of pathogen-related infections should be reviewed, and new strategies should be developed for the diagnosis of mixed *Candida* spp. infections.

Author contributions SHA: collecting resources, preparing figures, and writing up. GEHO: conceptualization. KE: review final draft of manuscript. HHA: language editing.

Funding No funding was received to assist with the preparation of this manuscript.

Data availability Data will be made available on reasonable request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical approval Not applicable.

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