Diploma thesis

Evaluation of new multiplex PCR-based assays for the detection of clinically relevant Candida species

submitted by

Melina Jobstmann

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Diagnostic and Research Institute of Hygiene, Microbiology and Environmental Medicine

First supervisor

Univ.-Prof. Dr. med. univ. Harald H. Kessler

Second supervisor

Priv.-Doz. Dr. med. univ. Dr. scient. med. Jürgen Prattes

Graz, 26.9.2019

I declare that I have written this diploma thesis independently, that I have not used other than the sources/ resources cited, and that I have explicitly marked all material, which has been quoted either literally or by content from the sources used.

Graz, am 26.9.2019 *Melina Jobstmann eh.*

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Abstract

Background: Blood cultures have been considered as diagnostic gold standard for candidemia. Compared to cultures, assays based on polymerase chain reaction (PCR) may be useful to shorten the time to diagnosis of invasive candidiasis and to initiate antifungal therapy. Recently, the new *Cand*ID® and *Cand*ID PLUS® kits (OLM Diagnostics) for molecular detection of different *Candida spp.* have been developed.
Objectives: The analytical and clinical performance of the new kits was investigated. Reference material and clinical specimens were used. Furthermore, the time-to-result required for the new assays was compared to the current gold standard.
Materials and Methods: Nucleic acid extraction was performed on the EMAG® platform. Real-time PCR (qPCR) and detection were performed on the LightCycler® 480 II instrument. The new *Cand*ID® and *Cand*ID PLUS® kits are based on multiplex qPCR providing detection of three different *Candida spp.* each and an internal control: *Candida albicans, Candida glabrata, Candida parapsilosis* with the *Cand*ID® and *Candida tropicalis, Candida krusei, Candida dubliniensis* with the *Cand*ID PLUS®. The accuracy of the new kits was determined utilizing the Quality Control for Molecular Diagnostics (QCMD) 2018 *Candida spp.* EQA Programme. The clinical performance of the new kits was studied with specimens obtained from patients with culture-proven candidemia (n=23; EDTA whole blood samples) and patients with bacteremia but no candidemia (n=31; plasma samples).
Results: When the QCMD panel was tested with the new kits, all members were correctly identified with the new PCR assays. Two EDTA whole blood samples obtained from patients with candidemia were found to be inhibited and thus excluded from further analysis. In patients with candidemia, 14 of 21 samples (67%) gave a positive result with all *Candida spp*. being identified correctly. In patients with bacteremia, 1 of 31 samples gave a positive result with the *Cand*ID® assay.
Conclusions: Detection of clinically relevant *Candida spp.* with the *Cand*ID® and *Cand*ID PLUS® kits shows a comparable specificity but an inferior sensitivity when compared to blood culture. Due to the shorter time to diagnosis, it may be useful as additional diagnostic tool allowing faster diagnosis and quicker start of antifungal therapy.

Kurzfassung

Hintergrund: Die Blutkultur gilt als diagnostischer Goldstandard für Candidämie. Im Vergleich zur Kultur können Tests, die auf Polymerasekettenreaktion (PCR) basieren, nützlich sein, um die Zeit bis zur Diagnose einer invasiven Candidose zu verkürzen und eine Antimykotika-Therapie einzuleiten. Kürzlich wurden die neuen *Cand*ID® und *Cand*ID PLUS® Kits (OLM Diagnostics) zum molekularen Nachweis verschiedener *Candida spp.* entwickelt. In dieser Studie wurden die analytische und klinische Leistung der neuen Kits untersucht. Referenzmaterial und klinische Proben wurden verwendet. Außerdem wurde die Gesamtzeit für die Analyse mit der des aktuellen Goldstandards verglichen.

Materialien und Methoden: Die DNA wurde auf der EMAG® Plattform extrahiert. Die real-time PCR (qPCR) und der Nachweis mit den *Cand*ID® und *Cand*ID PLUS® Kits wurden mit dem Light Cycler® 480 II Instrument durchgeführt. Beide Kits basieren auf multiplex qPCR und ermöglichen den Nachweis von jeweils drei verschiedenen Candida-Arten: *Candida albicans, Candida glabrata, Candida parapsilosis* mit dem *Cand*ID® Kit und *Candida tropicalis, Candida krusei, Candida dubliniensis* mit dem *Cand*ID PLUS® Kit. Die analytische Leistung wurde unter Verwendung des Quality Control for Molecular Diagnostics (QCMD) 2018 *Candida spp*. EQA Programms bestimmt. Die klinische Leistung wurde mit Proben aus zwei Gruppen von PatientInnen bestimmt: PatientInnen mit der Diagnose Candidämie aufgrund des Blutkulturbefundes (n = 23; EDTA-Vollblutproben) und PatientInnen mit Bakteriämie aber ohne Candidämie (n = 31; Plasmaproben).

Ergebnisse: Alle Referenzproben wurden mit den neuen PCR-Tests korrekt identifiziert. Zwei EDTA-Vollblutproben von PatientInnen mit Candidämie zeigten eine Inhibierung und wurden deshalb von der weiteren Analyse ausgeschlossen. Die neuen PCR-Tests lieferten bei 14 von 21 Candidämie-Proben (67%) ein positives Ergebnis und alle *Candida spp*. wurden richtig erkannt. Von den 31 Bakteriämie-Proben ergab eine mit dem *Cand*ID® Kit ein positives Ergebnis.

Fazit: Der Nachweis klinisch relevanter *Candida spp*. mit den neuen Kits zeigt im Vergleich zur Blutkultur eine ähnliche Spezifität, jedoch eine geringere Sensitivität. Aufgrund der kürzeren Gesamtzeit für die Analyse können sie als zusätzliches Diagnoseverfahren nützlich sein, um einen schnelleren Nachweis und damit einen rascheren Beginn der Antimykotikatherapie zu ermöglichen.

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1. Introduction

1.1 History

Written descriptions of oral lesions that were probably thrush date to the time of Hippocrates and Galen. In 1839, Bernhard von Langenbeck found fungi in oral lesions of a patient. In 1861, Friedrich Albert Zenker described the first well-documented case of deep-seated Candida. The first case of Candida-induced endocarditis was described in 1940 (1).

The most interesting period in the history of Candida infections began in the 1940s, when widespread use of antibiotics was introduced. Since then, previously undocumented manifestations of Candida infections have occurred, and the incidence of practically all forms of Candida infections has increased sharply (1). The development of modern medicine has been important for the increasing prevalence of Candida infections because of new therapeutic methods such as organ transplants and the increase of immunosuppressed patients and those needing intensive care in general. Another reason for the significant progression has been the increasing incidence of HIV infections in the 1980s.

1.2 Morphology

Candida (C.) is a genus of yeast which are single-celled eukaryotes that mostly have an oval shape called blastospore. Candida reproduces by budding but some yeasts also perform sexual reproduction like *C. krusei*. It grows well in vented routine blood cultures and on agar plates and does not require special fungal media for cultivation (1).

1.2.1 Microscopy

Direct microscopic examination of clinical specimens containing *Candida spp.* reveals budding yeast cells (blastoconidia) 2 to 4 µm in diameter and/or pseudohyphae showing regular points of constriction, resembling links of sausages (Figure 1). True septate hyphae (filamentation) may also be produced by *C. albicans* and *C. dubliniensis.* The blastoconidia, pseudohyphae, and hyphae are gram positive. The approximate number of such forms should be reported, because the

presence of large numbers in a fresh clinical specimen may have diagnostic significance. Microscopically, *C. glabrata* blastoconidia are notably smaller (at 1-4 µm) than those of other medically significant *Candida spp.* (2).

Figure 1. Yeast cells, pseudohyphae, and hyphae (from left to right) (3).

1.2.2 Macroscopy

Most *Candida spp.* produce smooth, creamy, white colonies that may resemble staphylococcal colonies (Figure 2). In a few cases, they also may establish dry, wrinkled, dull colonies. Yeasts have a capsule resulting in a shiny and/or mucoid colonial appearance. They may display bright pigments or appear hyaline or melanized (dematiaceous) (2).

Figure 2. Candida colonies with typical spikes on blood agar; large colonies at the right top are other microorganisms (4).

1.2.3 Reproduction

In general, the yeasts reproduce asexually by blastoconidia formation (budding). The process begins with a weakening and subsequent outpouching of the yeast cell wall. This process continues until the bud, or daughter cell, is completely formed. The cytoplasm of the bud is contiguous with the cytoplasm for the original cell. Finally, a cell wall septum is established between mother and daughter cells. The daughter cell eventually detaches from the mother cell, and a residual defect occurs at the budding site (i.e. a bud scar) (2).

1.3 *Candida spp.*

There are more than 200 different species of the genus Candida which are very heterogenous and vary in their clinical significance. The frequency of *Candida spp*. depends on the institution and geographic distribution. *Candida spp.* are responsible

for the most commonly encountered opportunistic fungal infections and candidemia has often been cited as the fourth most common bloodstream infection in ICU patients (2, 5).

1.3.1 *C. albicans*

C. albicans represents the most commonly species of the genus Candida being able to cause infection in humans (Figure 3). *C. albicans* is a unicellular yeast of the Cryptococcaceae family with a single bud. As a member of the normal microflora, it is present on the skin and the mucous membranes of the upper respiratory tract, gastrointestinal tract and female genital tracts. It reproduces by asexual budding and grows rapidly at 25–37°C and within a pH range of 2–8 (6).

C. albicans grows as either yeast or hyphal cells. This characteristic is known as dimorphism. The yeast form is believed to be harmless, but the hyphae form is usually associated with invasion into the host tissue. This transition from a benign yeast type to highly invasive hyphae type depends on changes in the host defenses (6).

There have been more than 100 synonyms for *Candida albicans*. The two that have persisted are *Monilia albicans* and the well-known name *Candida albicans. Candida* comes from candidus, the Latin word for white, and *albicans* is the present participle of the Latin word albico, meaning becoming white (1, 7).

Infection starts with adherence of a mannoprotein component, the extracellular polymeric material that coats the surface of *C. albicans*. Then, *C. albicans* produces a range of proteinases and phospholipases which are particularly concentrated on the tips of the fungal hyphae that disrupt the cell membrane resulting in invasion of the pathogen into host tissue. Importantly, *C. albicans* has developed the capacity to vary its phenotype by spontaneously generating variants within infecting populations (6).

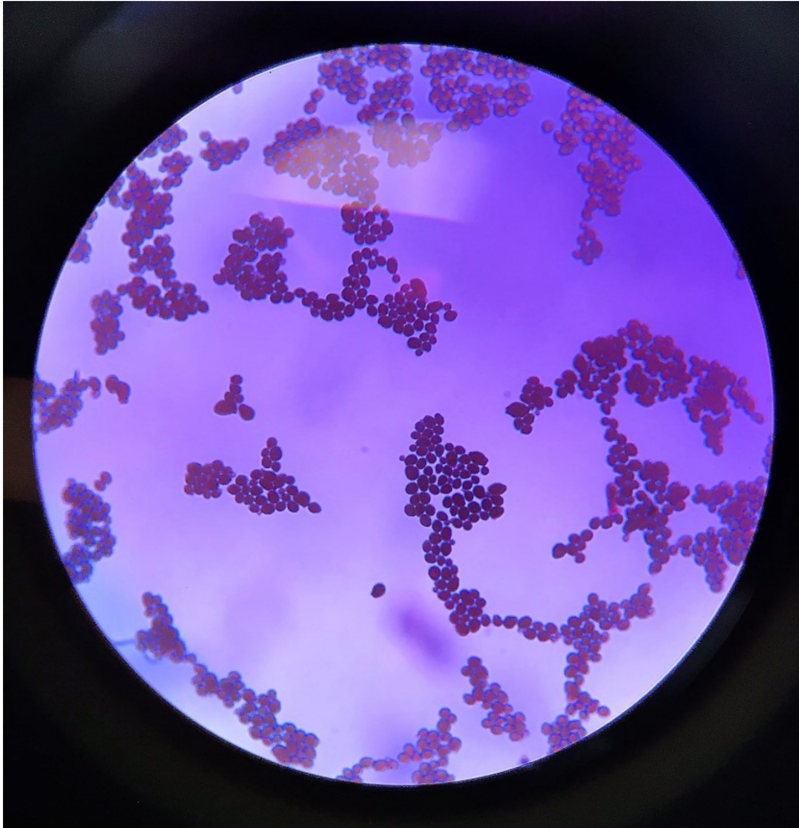

Figure 3. Microscopic view of Gram stained *C. albicans* (image source: Melina Jobstmann).

1.3.2 Non-albicans Candida

The most significant species of non-albicans Candida include *C. glabrata, C. parapsilosis, C. tropicalis, C. krusei,* and *C. dubliniensis.* The species distribution has changed over the past decades. Whereas *C. albicans* had previously been the dominating pathogen, this species today accounts for only half of the isolates detected in many surveys (5).

1.3.2.1 *C. glabrata*

C. glabrata is a haploid yeast that has aroused poor clinical interest in the past decades being mostly considered as a non-pathogenic commensal of the normal human microbiota. However, as a result of the diffusion of broad-spectrum antifungal prophylaxis and immunosuppressive therapies, this species has been increasingly implicated in several human diseases and is a common cause of bloodstream infections. Generally, *C.glabrata* is considered a non-dismorphic fungus, therefore it does not form pseudohyphae on corn meal agar, grows as small,

elliptical budding yeast on common culture media and has very limited repertoire of sugar utilization making its identification easier (8).

1.3.2.2 *C. parapsilosis*

C. parapsilosis is an opportunistic human pathogen, whose incidence in clinically diagnosed candidiasis has dramatically increased in the past two decades. Because of its worldwide distribution and its relevant role as etiological agent of invasive candidiasis many studies focused on this pathogenic yeast. *C. parapsilosis* shows intrinsic and unusual heterogeneity, therefore it was decided to treat them as cryptic complex to which the two new proposed species *C. orthopsilosis* and *C. metapsilosis* belong (8). Despite its low virulence, *C. parapsilosis* can appear in certain clinical settings owing to its ability to adhere to medical devices and its tendency to colonize human skin, characteristics that enable nosocomial outbreaks (5).

1.3.2.3 *C. tropicalis*

C. tropicalis has been shown to be prevalent in patients with hematologic malignancies, especially those who are neutropenic. Mouse models of infection and human studies have shown *C. tropicalis* in the submucosa of the gastrointestinal tract surrounded by necrotic tissue. This may indicate that the organism can invade the gastrointestinal tract efficiently, particularly in oncology patients. This phenomenon most likely is a result of the expression and secretion of aspartyl proteases and tropiase (acid proteinase), a virulence factor found in Candida organisms. (2)

1.3.2.4 *C. krusei*

C. krusei has been described as a causative agent of disseminated fungal infections in susceptible patients, resulting in the lowest 90-day survival rates among common *Candida spp*. The fact that infection with *C. krusei* is highly uncommon except in patients with severe immunodeficiency and prior exposure to an azole should be noticed. Although the prevalence of *C. krusei* remains low (approx. 2%) among yeast infections, identification of this species is essential to proper clinical management of the patient because *C. krusei* is inherently resistant to the azole class of antifungal drugs (5, 9).

1.3.2.5 *C. dubliniensis*

In 1995, the phylogeny of *C. albicans* underwent important changes due to the recognition of a new closely related pathogenic species called *C. dubliniensis.* This discovery had important diagnostic consequences since this new species, like *C. albicans*, produces chlamydospores and germ-tubes and can still be misidentified as such by using biochemical tests and/or conventional identification methods. However, *C. dubliniensis* can be easily distinguished from *C. albicans* by a number of molecular methods. Current epidemiological data show that *C. dubliniensis* is much more prevalent in oropharyngeal infections than in invasive candidiasis and it is rarely implicated in vaginal infections (8).

1.4 Pathogenesis

Many Candida species (especially *C. albicans*) are commensals of the human mucosa. Usually they are harmless and do not cause any infection. Immunological defense mechanisms involving especially neutrophil granulocytes and macrophages are of importance in maintaining resistance to candidiasis. However, certain circumstances can lead to an overgrowth of the fungus and cause health problems. Conditions that increase the risk of candidiasis are:

- Antibiotic usage (reduction of the physiological bacterial flora)
- Hormonal contraception or gravidity (pH elevation or estrogen overproduction)
- Skin damage (e.g. skin burns)
- Immunosuppression because of illness (e.g. AIDS, leukemia, cancer)
- Iatrogenic immunosuppression (e.g. transplantation, radiotherapy, cytostatic drugs, corticosteroids)
- Metabolic disorder (e.g. diabetes)

1.4.1 Invasive candidiasis

Invasive candidiasis is generally caused by a change in the concentration and distribution of normal flora combined with an alteration of the barrier function of the mucous membranes. Critical illness and critical care therapies lead to dysbiosis and disruption of the normal barrier mechanisms, resulting in translocation of flora,

including Candida. Moreover, the pathogenicity of Candida is enhanced by its ability to adhere to prosthetic surfaces and form biofilms (10). The main risk factors for invasive Candidiasis are:

- Diabetes mellitus
- Neutropenia
- Renal insufficiency
- Surgery, mainly abdominal
- Pancreatitis
- Use of broad-spectrum antibiotics
- Parenteral nutrition
- Hemodialysis
- Mechanical ventilation
- Presence of central venous catheters
- Immunosuppressive agents

1.5 Candidiasis

1.5.1 Superficial Candida infections

Superficial Candida infections are common and include vaginal and oral candidiasis, skin and nail infections, which can arise in warm and moist areas such as skin folds and as complication of antibiotic therapy because of the reduction of the bacterial flora (11).

1.5.1.1 Thrush

The term “thrush” is applied to a specific form of oral candidiasis characterized by creamy white, curd-like patches on the tongue and on other oral mucosal surfaces (Figure 4). The patches are removable by scraping and leave a raw, bleeding and painful surface. The patches are actually a pseudomembrane consisting of Candida, desquamated epithelial cells, leucocytes, bacteria, keratin, necrotic tissue, and food debris (1). Thrush can be seen in newborns, patients with HIV infection, individuals with diabetes, patients undergoing chemotherapy etc. (2).

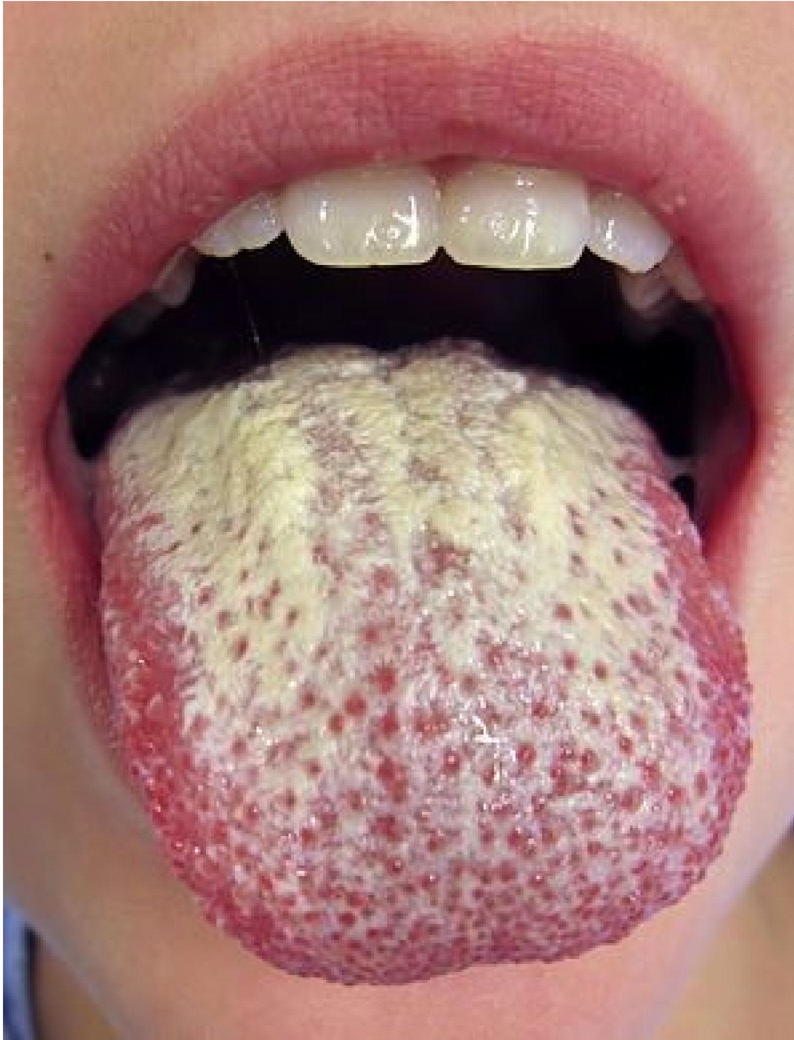

Figure 4. Oral candidiasis (thrush) on a patient's tongue (12).

1.5.1.2 Candida esophagitis

Esophageal disease was believed to occur by direct spread from oral disease, but reviews have shown that Candida esophagitis may occur frequently without thrush. The most common symptoms include painful swallowing, a feeling of obstruction on swallowing, and substernal chest pain. Nausea and vomiting also occur. The diagnosis is made definitively by biopsy during endoscopy. Candida esophagitis is often associated with treatment of malignancy of the hematopoietic or lymphatic systems and in AIDS patients (1).

1.5.1.3 Candida vaginitis

This common infection is most frequently seen in a setting of diabetes mellitus, antibiotic therapy, and pregnancy. In addition, the use of birth control pills may be a predisposing factor although this association is controversial. However, estimates

are that 75% of women have an episode of candida vaginitis during their lifetime and many have no recognizable underlying predisposing factor. Although Candida-induced vaginitis may be accompanied by a thick, curd-like discharge, low discharge may instead characterize the infection. Edema and intense pruritus of the vulva are almost always present (1).

1.5.2 Invasive Candida infections

Invasive Candida infection usually arise from the patient's own commensal flora following surgery, use of broad-spectrum antibiotics, or intravascular catheters and can affect any organ of the body (Figure 5). Candidemia may result in abscesses in various organs (e.g. brain, liver, spleen). These infections occur primarily in immunocompromised patients. Candida can also colonize prosthetic materials (e.g. intravascular or peritoneal dialysis catheters) resulting in bloodstream infections and peritonitis. In addition, *Candida spp.* are a rare cause of endocarditis (11). Especially patients-at-risk develop invasive Candida infections showing continuing fever (>38 C) under broad-spectrum antibiotic treatment. Organ-specific symptoms are caused through colonization of different organs showing a high mortality.

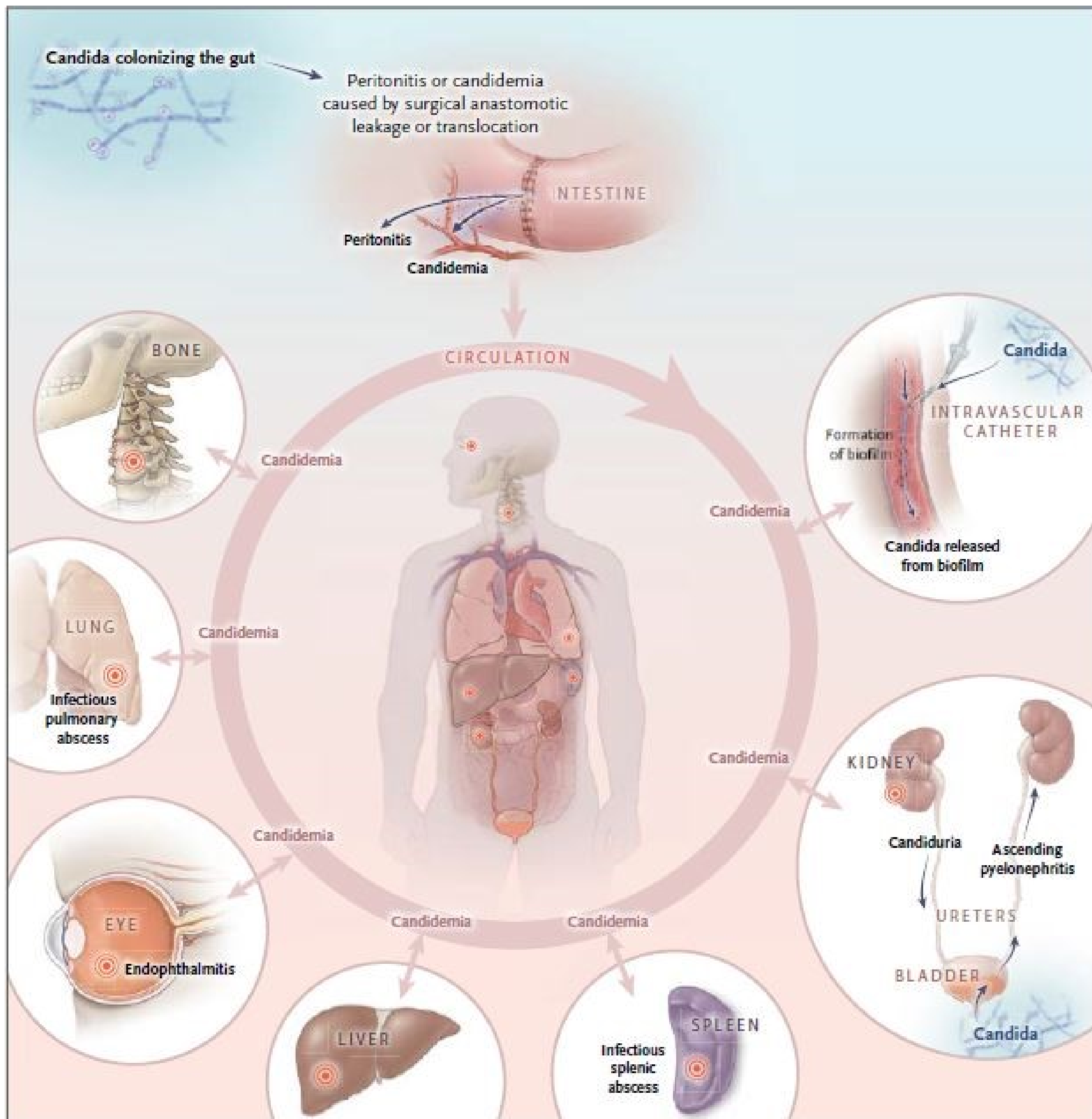

Figure 5. Pathogenesis and organ manifestations of invasive candidiasis (5).

1.6 Diagnostics

Cultures of blood or other material collected under sterile conditions have long been considered diagnostic gold standards for invasive candidiasis. Nonculture diagnostic tests, such as antigen, antibody, or β-D-glucan detection assays, and polymerase chain reaction (PCR) are now entering clinical practice in addition to cultures (Table 1). If used and interpreted correctly, these tests can identify more patients with invasive candidiasis and lead to better direct antifungal therapy (13).

1.6.1 Blood culture

The overall sensitivity of blood cultures for diagnosing invasive candidiasis is roughly 50%. The limit of detection of blood cultures is ≤1 colony-forming unit/ml. As such, blood cultures should be positive during the vast majority of active Candida bloodstream infections. They may be negative in cases of extremely low-level candidemia, intermittent candidemia, deep-seated candidiasis in absence of candidemia or deep-seated candidiasis that persists after sterilization of the bloodstream. Blood cultures are limited by slow turnaround times (median time to positivity 2-3 days, ranging from 1 to ≥7 days) and the fact that they may become positive relatively late in the disease course (13).

1.6.2 Antigen and antibody detection

Candida antigen and anti-Candida antibody detection has gained greater acceptance in Europe than the United States. In general, antigen detection is limited by rapid clearance from the bloodstream. Serum immunoglobulin G (IgG) responses against specific antigens have typically performed better than immunoglobulin M (IgM) responses. The best studied test is a combined mannan/antimannan antibody assay (13). The sensitivity and specificity of mannan-Ag alone have been disappointing in several studies but the combination of mannan-Ag and anti-mannan-Ab assays significantly increases the sensitivity and specificity of the test. The best sensitivity results have been reported with *C. albicans*, *C. glabrata* and *C. tropicalis*, whereas disappointing results have been reported in *C. parapsilosis* and *C. guilliermondii* infections (14).

1.6.3 β-D-Glucan detection

β-D-glucan (BDG) is a cell wall constituent of Candida species and several other fungi. True-positive results are not specific for invasive candidiasis but rather suggest the possibility of an invasive fungal infection. The high negative predictive value of the test allows its use to exclude invasive candidiasis. BDG detection can identify cases of invasive candidiasis days to weeks prior to positive blood cultures (by a median of 5-8 days), and shorten the time to initiation of antifungal therapy. In addition, repeated measurements can increase the diagnosis accuracy.

The major concern about BDG detection is the potential for poor specificity and false positivity. Causes of false positivity include other fungal and bacterial infections and many therapeutic interventions, such as antibiotics, hemodialysis, surgical gauze and other material containing glucan, blood products, immunoglobulins and many more. Furthermore, the sensitivity of the test seems to vary by Candida species, with the lowest sensitivity for *C. parapsilosis* (13, 14).

1.6.4 Polymerase chain reaction

Candida PCR shares many of the potential benefits and shortcomings of BDG detection. Compared to cultures, assays based on polymerase chain reaction (PCR) of various blood fractions have been shown to shorten the time to diagnosis of invasive candidiasis and initiation of antifungal therapy. Reports in ICU patients have demonstrated good sensitivity, specificity and predictive values. PCR has potential advantages over β-D-glucan or antigen-antibody assays, including the capacity for species identification, detection of molecular markers for drug resistance, and multiplex formatting.

A major limitation of PCR is the lack of standardized methodologies and multicenter validation of assay performance. High costs and the labor-intensive nature of its use are further limitations. No officially standardized PCR test is yet available and the usefulness of PCR as an early marker of invasive candidiasis is a subject of debate(13, 14).

In general, molecular assays for detection of *Candida spp.* employ the real-time polymerase chain reaction (qPCR) technology today. The most frequently used assay format utilizes the hydrolysis probe technique. The qPCR consists of three steps (Figure 6):

1. Denaturation: The double stranded DNA is separated into two single strands as the sample is briefly heated to 95°C by the thermocycler.
2. Annealing: The temperature is reduced to 60°C causing forward and reverse primers to hybridize to any complementary target DNA present. The same reaction mixture also contains fluorogenic probes which consist of target-specific DNA oligonucleotides with a fluorescent reporter dye attached to the 5´ ends and a quencher dye at the 3´ ends. While the probe is intact, the quencher dye reduces the fluorescence emitted by the reporter dye.

3. Polymerization/hydrolysis of probes: The polymerization starts from the primers and once the segment of the hydrolysis probe is reached, the 5' nuclease activity of the Taq polymerase causes the displacement of the hybridized bound probes resulting in the physical separation of the reporters and quenchers. Now the fluorescence emitted by the reporter dye is no longer inhibited by the quencher. This results in the emission of light at a fluorophore-specific wavelength which can be detected in the appropriate channel of the qPCR instrument.

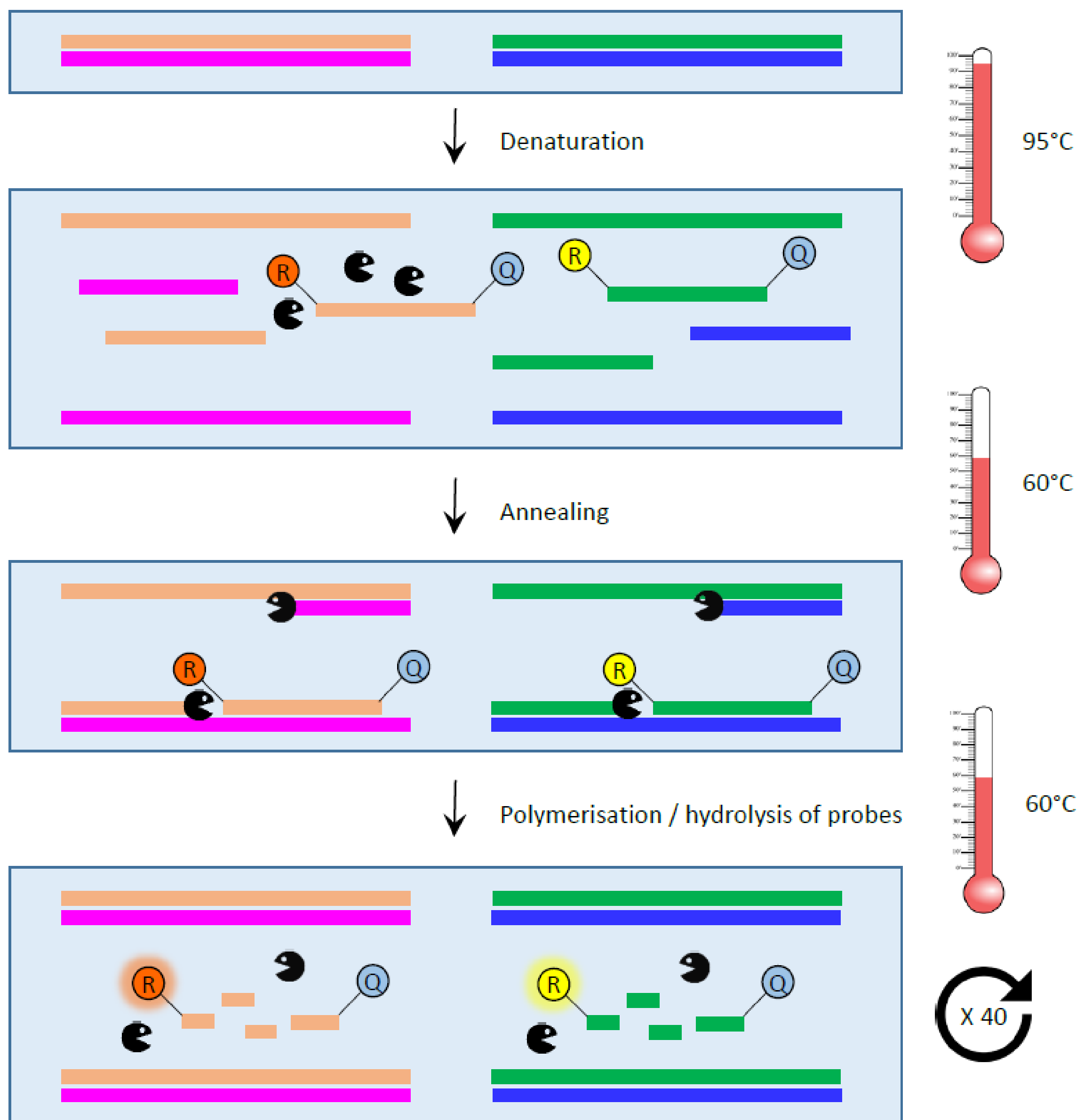

Figure 6. Principle of the hydrolysis probe-based test format (image source: Daniel Ostermann)
Orange and pink lines = double stranded genomic DNA; Blue and green lines = Internal extraction control; Short colored lines = forward and reverse primers; Circled "R" = 5'-fluorescent reporter dye; Circled "Q" = quencher; Black figures = Taq polymerase

Table 1
Non-culture based diagnostics (based on IDSA Candida guidelines 2016) (14).

Method or marker	Sensitivity/ specificity	Potential advantages	Potential limitations
Candida PCR	95%/92% (suspected); 85%/38% (probable)	• Shorter time to diagnosis • Species identification • Detection of resistance markers • Detection of deep-seated candidiasis	• Cost, inconvenience • Lack of universally standardized methods (e.g. specimen type) or performance validation
Mannan-Ag and anti-mannan Ab	58%/93% (mannan-Ag); 59%/83% (anti-mannan Ig); 83%/86% (combined)	• Best when used together and for detecting *C. albicans, C. glabrata* or *C. tropicalis*	• Limited sensitivity/specificity when used individually and for detecting *C. parapsilosis* and *C. guilliermondii* • Uncertain reliability in immunocompromised hosts, uncertain utility for deep-seated candidiasis
BDG	75%–80%/80%	• Pan-fungal marker for patients at risk for other systemic infections (e.g. with *Aspergillus spp*. or Pneumocystis jirovecii, in HSCT recipients) • Detection of deep-seated candidiasis • High negative predictive value • Can detect infection days or weeks in advance of culture-based diagnosis	• Prophylactic or empirical antifungal treatment may impact test performance • Lower sensitivity for *C. parapsilosis* • False-positive results higher for patients in ICU, with colonization, other systemic infections, multiple therapeutic interventions; may require more than one consecutive positive result

1.7 Treatment

Candidemia requires treatment with an antifungal agent and it should never be assumed that removal of a catheter alone is a sufficient therapy for. Furthermore, prompt initiation of therapy is crucial. Untreated candidemia has an overall mortality rate of more than 60%. With treatment, the overall mortality of candidemia is approximately 30 to 40% (15).

1.7.1 Factors to consider

It is important to determine the Candida species because some *C. glabrata* isolates are resistant to fluconazole, while all *C. krusei* isolates are resistant to fluconazole. In addition, the minimal inhibitory concentrations for *C. parapsilosis* with the echinocandins are higher than for other Candida species. Risk factors for infection with fluconazole-resistant Candida species include neutropenia, recent azole use, and others (15). In addition to that, it is important to distinguish between neutropenic and nonneutropenic patients regarding the choice of the initial agent and the dosage suggested (Table 2).

Table 2
Treatment for Candidemia (based on the 2016 IDSA recommendations) (10).

Non neutropenic patients	Neutropenic patients
First-line therapy	
Echinocandins: Caspofungin: 70 mg initially, then 50 mg daily Micafungin: 100 mg daily Anidulafungin: 200 mg initially, then 100 mg daily	
Second-line therapy	
Fluconazole: 12 mg/kg initially, then 6 mg/kg daily (not in critically ill and only when resistance is unlikely). When susceptibility is established (after 5-7 days), step-down therapy. Negative repeated blood cultures must be documented. For *C. glabrata,* 12 mg/kg daily.[a]	Lipid formulation amphotericin B: 3-5 mg/kg
Alternatives	
Lipid formulation amphotericin B: 3-5 mg/kg in case of intolerance or resistance to other agents	Fluconazole (see at non neutropenic patients), not in critically ill and only if no prior exposure. Voriconazole 6 mg/kg for 2 doses and then 3-4 mg/kg. Both drugs can be used as step-down therapy.

[a]For fluconazole-resistant *C. glabrata*, amphotericin B deoxycholate, 0.3–0.6 mg/kg daily for 1–7 days OR oral flucytosine, 25 mg/kg 4 times daily for 7–10 days.

1.7.2 Antifungal agents

The most common antifungal agents used for the treatment of candidemia are the echinocandins (caspofungin, micafungin, anidulafungin) and fluconazole. Formulations of amphotericin B are given less often due to the risk of toxicity. Both

the echinocandins and the azoles are better tolerated than amphotericin B formulations (15). Combination therapy is not recommended for candidiasis.

Table 3
Spectrum of activity of available systemic antifungal agents. The table incorporates the present ESCMID/IDSA (European Society of Clinical Microbiology and Infectious Diseases/Infectious Diseases Society of America) guidelines (10).

	Polyenes	FC	Triazoles					Echinocandins		
	AMB*	5FC*	FLU*	ITR	VOR*	POS	ISA	CAS	MICA	ANI
C. albicans	++	++	++	++	++	++	++	++	++	++
C. glabrata	++	++	+	+	++	++	++	+	+	+
C. parapsilosis	++	++	++	++	++	++	++	++	++	++
C. tropicalis	++	++	++	++	++	++	++	++	++	++
C. krusei	++	+	-	+	++	++	++	++	++	++
C. lusitaniae	-	++	++	++	++	++	++	++	++	++
Cryptococcus neoformans	++	++	++	++	++	++	++	-	-	-

5FC, flucytosine; AMB, amphotericin B; ANI, anidulafungin; CAS, caspofungin; FLU, fluconazole; ISA, isavuconazole; ITR, itraconazole; MICA, micafungin; POS, posaconazole; VOR, voriconazole.
Green background, potent antifungal activity; orange background, increasing fungal resistance; red background, definitive fungal resistance; CNS, central nervous system.
*Good penetration in CNS, eye.

1.7.3 Duration of therapy

The recommended duration of therapy for candidemia without complications is for 2 weeks after documented clearance of Candida species from the bloodstream and resolution of signs attributable to candidemia (13). A longer duration of therapy and consultation with an infectious disease specialist are suggested in patients who have metastatic foci of infection, such as endophthalmitis or endocarditis. Blood cultures should be performed daily or every other day after initiating therapy in order to determine the date of sterilization. If blood cultures remain positive, then a search for a metastatic focus, such as an abscess or endocarditis, must be undertaken. In addition, all patients should have resolution of symptoms attributable to candidemia and resolution of neutropenia (e.g., absolute neutrophil count >500 cells/μL and a consistent increasing trend) before antifungal therapy is discontinued (15).

2. Objectives

Recently, two new assays for detection of different Candida species have been brought on the market. They are based on multiplex qPCR and able to detect 3 different Candida species each. One of the assays is able to identify *Candida albicans, Candida glabrata* and *Candida parapsilosis*, the other *Candida tropicalis, Candida krusei* and *Candida dubliniensis*

Goals of this study:

(1) To evaluate the analytical performance of two new molecular assays for detection of different Candida species using reference material.

(2) To evaluate the clinical performance of two new molecular assays for detection of different Candida species and compare results obtained with those obtained from blood culture as gold standard.

(3) To estimate time-to-result required for the new assays in comparison with the current gold standard.

3. Materials and Methods

3.1 Study design

3.1.1 Analytical performance

The accuracy of the *Cand*ID® and *Cand*ID PLUS® kits (OLM Diagnostics, Newcastle Upon Tyne, England) was determined utilizing the Quality Control for Molecular Diagnostics (QCMD) 2018 *Candida spp*. EQA Programme. The panel consisted of 10 members including *C. albicans*, *C. auris*, *C. glabrata*, *C. krusei*, and vials without *Candida spp*. Characteristics of the panel is shown in Table 4.

Table 4
Characteristics of the proficiency panel used in this study.

Vial no.	Sample content	Matrix
1	*C. albicans*	Plasma
2	*C. albicans, C. glabrata*	Plasma
3	*C. glabrata*	Plasma
4	*C. albicans*	Plasma
5	Negative	Plasma
6	*C. albicans*	Synthetic BAL
7	*C. auris*	Plasma
8	*C. albicans*	Plasma
9	*C. krusei*	Plasma
10	*Saccharomyces cervisiae*	Plasma

BAL, bronchoalveolar lavage.

3.1.2 Clinical performance

In this retrospective study, the clinical performance of the new kits was determined with specimens collected from two groups of patients based on results obtained from blood culture (gold standard): 23 patients with candidemia and 31 patients without candidemia but bacteremia. The characteristics of the different groups are shown in Table 5. Whole blood samples from patients were collected at the same day as the corresponding blood cultures. Blood cultures were investigated immediately after arrival in the laboratory while EDTA whole blood samples were stored at -80° C until analysis.

Table 5
Main characteristics of patients with and without candidemia included in the study based on results obtained by blood culture.

	Patients with candidemia	Patients without candidemia
No. of patients	23	31
Matrix	EDTA whole blood	Plasma
Mean age, years (age range)	67 (20-89)	62 (23-91)
Female : male	9 : 14	9 : 22

3.1.3 Lab flow analysis

For the lab flow analysis, the turn-around time including hands-on time were estimated. Times required for the *Cand*ID® and *Cand*ID PLUS® kits were compared to those for blood culture.

3.2 Methods

3.2.1 Extraction of nucleic acids

Nucleic acid extraction was performed on the EMAG® using the magnetic particle technology for capture of nucleic acids platform (bioMérieux, Marcy-l´Etoile, France; Figure 7). All samples were extracted with the EMAG® accessory products

(bioMérieux) using the specific B protocol. The input volume was 250 µl. The extracted DNA was eluted automatically with 25 µl of elution buffer.

Figure 7. The EMAG® platform (image source: Melina Jobstmann).

3.2.2 Amplification and detection

For qPCR and detection with the *Cand*ID® and *Cand*ID PLUS® kits, 14 µl of the PCR mix and 6 µl of the extracted DNA were pipetted into a well of a PCR plate. Extracted controls were treated in the identical way. After sealing the plate, amplification and detection were performed on the Light Cycler® 480 II CE/IVD (Roche Diagnostics, Penzberg, Germany) instrument (Figure 8).

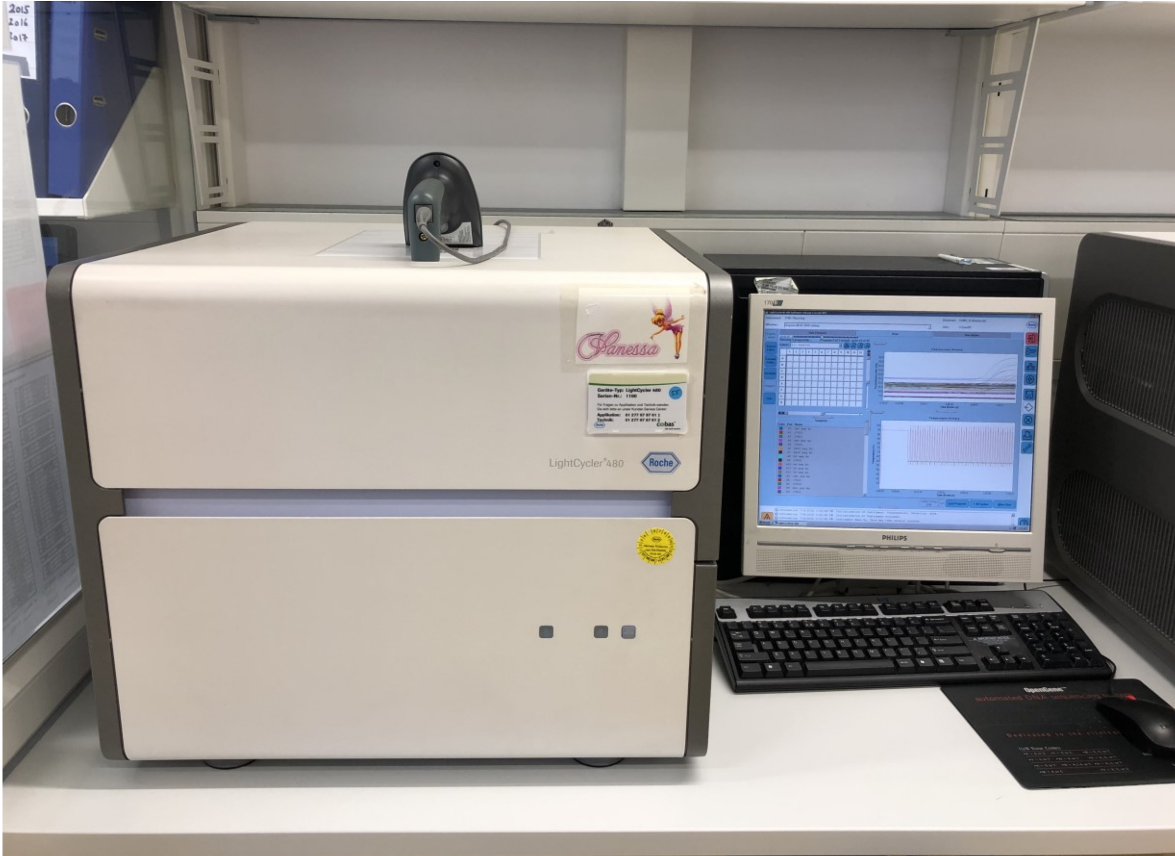

Figure 8. The Lightcycler 480 II instrument (image source: Melina Jobstmann).

The *Cand*ID® and *Cand*ID PLUS® kits include individual primer and probe designs for detection of three clinically relevant *Candida spp*. each and an internal control: *Candida albicans*, *Candida glabrata*, *Candida parapsilosis* with the *Cand*ID® and *Candida tropicalis*, *Candida krusei*, *Candida dubliniensis* with the *Cand*ID PLUS®. Both kits are based on the qPCR technique in combination with detection using a hydrolysis probe-based technique detecting amplified DNA's through different fluorescence channels.

3.2.3 Internal and external quality controls

To ensure the correctness of results obtained, each molecular assay must include appropriate quality controls. The *Cand*ID® and *Cand*ID PLUS® kits contain an internal quality control, the internal extraction control (IEC), and two external quality controls, one positive and one negative control.

The IEC is added to distinguish true negative samples from false negative ones, which may result from nucleic acid degradation, failure of nucleic acid extraction, PCR inhibition, non-specific probe hydrolysis, or qPCR instrument malfunction. The primers and probe necessary to detect the IEC are included in the multiplex primer and probe mix. The IEC template is added either to the DNA lysis/extraction buffer or to the sample once it has been resuspended in lysis buffer. Under optimized conditions, the IEC shows a threshold cycle (Cp) value of 25 or higher, with the variation depending on the efficiency of sample extraction and level of sample dilution.

Furthermore, the *Cand*ID® and *Cand*ID PLUS® kits include a positive control tube with a template specifying the different Candida species targets. The positive control is handled like a normal nucleic acid extract and indicates that the primers and probes for detecting Candida species are working properly in the run. Its use is recommended in every run as it provides more confidence in results if all samples are negative. The positive control does not need to be subjected to the nucleic acid extraction procedure. Care should be taken to avoid cross-contamination of other samples when adding the positive control to the run. Any risk can be minimized by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

To confirm the absence of contamination, the kits include the no template control that must also be included in each PCR run. For this reaction, RNase/DNase free water is used instead of template.

4. Results

4.1 Analytical performance

When accuracy was determined, 7 of 8 panel members containing *Candida spp*. were identified as positive (Table 6). As expected, the kits did not detect one sample containing *C. auris* (vial no.7) which is not included in the *Cand*ID® and *Cand*ID PLUS® kits. Furthermore, fungal species other than *Candida spp*. (vial no.10) was not detected by any of the kits. The negative panel member (vial no. 5) was correctly identified as negative. In that vial containing synthetic bronchoalveolar lavage as a matrix (vial no. 6), *C. albicans* was correctly detected; however, *C. parapsilosis* was found additionally.

Table 6
Results of accuracy testing utilizing the Quality Control for Molecular Diagnostics (QCMD) 2018 *Candida spp*. EQA Programme.

Vial no.	Sample content	Matrix	Result obtained*
1	*C. albicans*	Plasma	*C. albicans*
2	*C. albicans, C. glabrata*	Plasma	*C. albicans, C. glabrata*
3	*C. glabrata*	Plasma	*C. glabrata*
4	*C. albicans*	Plasma	*C. albicans*
5	Negative	Plasma	Negative
6	*C. albicans*	Synthetic BAL	*C. albicans, C. parapsilosis*
7	*C. auris*	Plasma	Negative
8	*C. albicans*	Plasma	*C. albicans*
9	*C. krusei*	Plasma	*C. krusei*
10	*Saccharomyces cervisiae*	Plasma	Negative

*Combined result obtained by the *Cand*ID® and *Cand*ID PLUS® plus kits.

4.2 Clinical Performance

With the new *Cand*ID® and *Cand*ID PLUS® kits, 2 EDTA whole blood samples obtained from patients with candidemia were found to be inhibited and thus excluded from further analysis.

In patients with candidemia 14 of 21 samples gave a positive result when employing the new PCR assays (Table 7, Figure 9, Figure 10). All *Candida spp*. in each of the 14 samples were correctly identified. The kits did not detect 5 samples containing *C. albicans* and 2 samples containing *C. glabrata*.

In patients without candidemia, 30 of 31 samples gave a negative result with the *Cand*ID® assays. One sample was found to be positive for *C. parapsilosis* when using the *Cand*ID® kit. This sample was positive for *Peptostreptococci* with blood culture.

When results obtained by the *Cand*ID® and *Cand*ID PLUS® kits were compared to those obtained by blood culture, 44 were found to be concordant and 8 discordant (one qPCR-positive result with negative culture and 7 qPCR-negative results with positive cultures; Table 8). Sensitivity and specificity for qPCRs were 66.7% and 96.8%, respectively.

Table 7
Results of samples of patients with culture-proven candidemia obtained with the new *Cand*ID® and *Cand*ID PLUS® kits.

Vial no.	Pathogens in blood culture	Result obtained with molecular assays*
1	*C. albicans*	Negative
2	*C. parapsilosis*	*C. parapsilosis*
3	*C. glabrata*	*C. glabrata*
4	*C. albicans*	*C. albicans*
5	*C. albicans*	*C. albicans*
6	*C. glabrata*	*C. glabrata*
7	*C. albicans*	*C. albicans*
8	*C. albicans*	*C. albicans*
9	*C. albicans*	Negative
10	*C. glabrata*	Negative
11	*C. albicans*	Negative
12	*C. glabrata*	*C. glabrata*
13	*C. albicans*	*C. albicans*
14	*C. albicans*	Negative
15	*C. albicans*	*C. albicans*
16	*C. albicans*	*C. albicans*
17	*C. albicans*	Negative
18	*C. albicans*	*C. albicans*
19	*C. albicans*	*C. albicans*
20	*C. glabrata*	Negative
21	*C. albicans, C. krusei*	*C. albicans, C. krusei*

*Combined result obtained by the *Cand*ID® and *Cand*ID PLUS® kits.

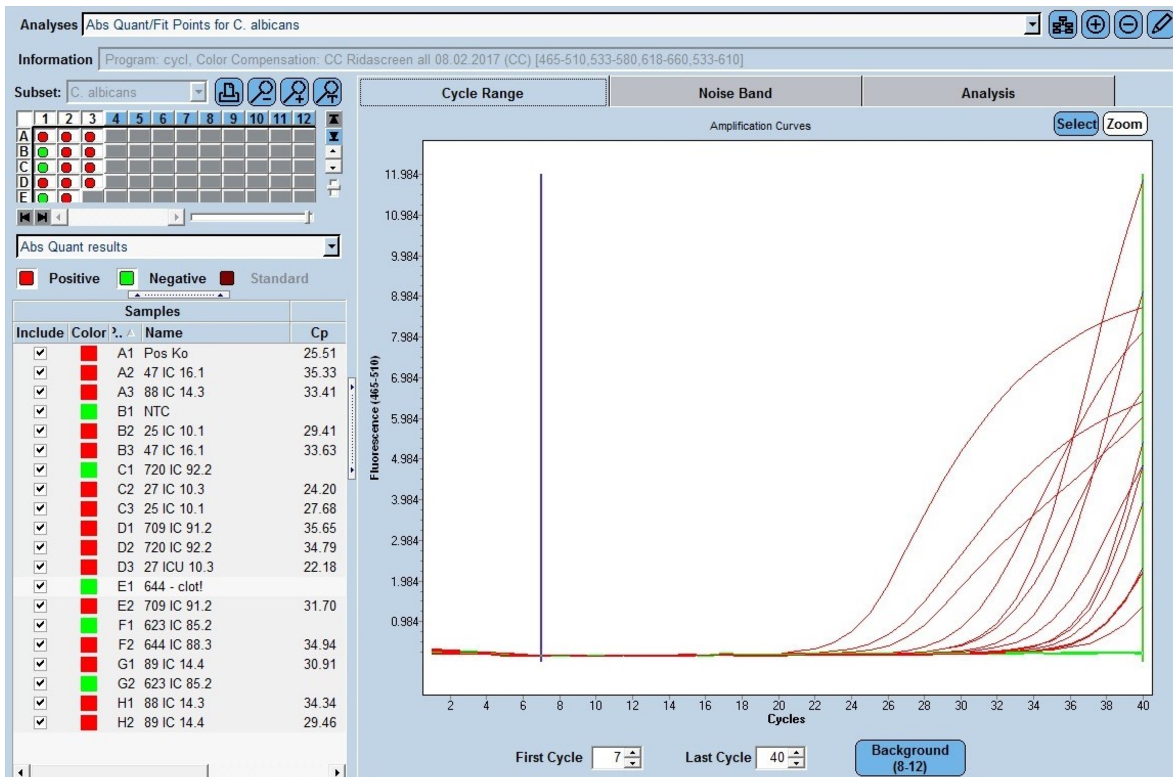

Figure 9. Lightcycler qPCR results for *C. albicans* with the *Cand*ID® kit (image source: Melina Jobstmann).

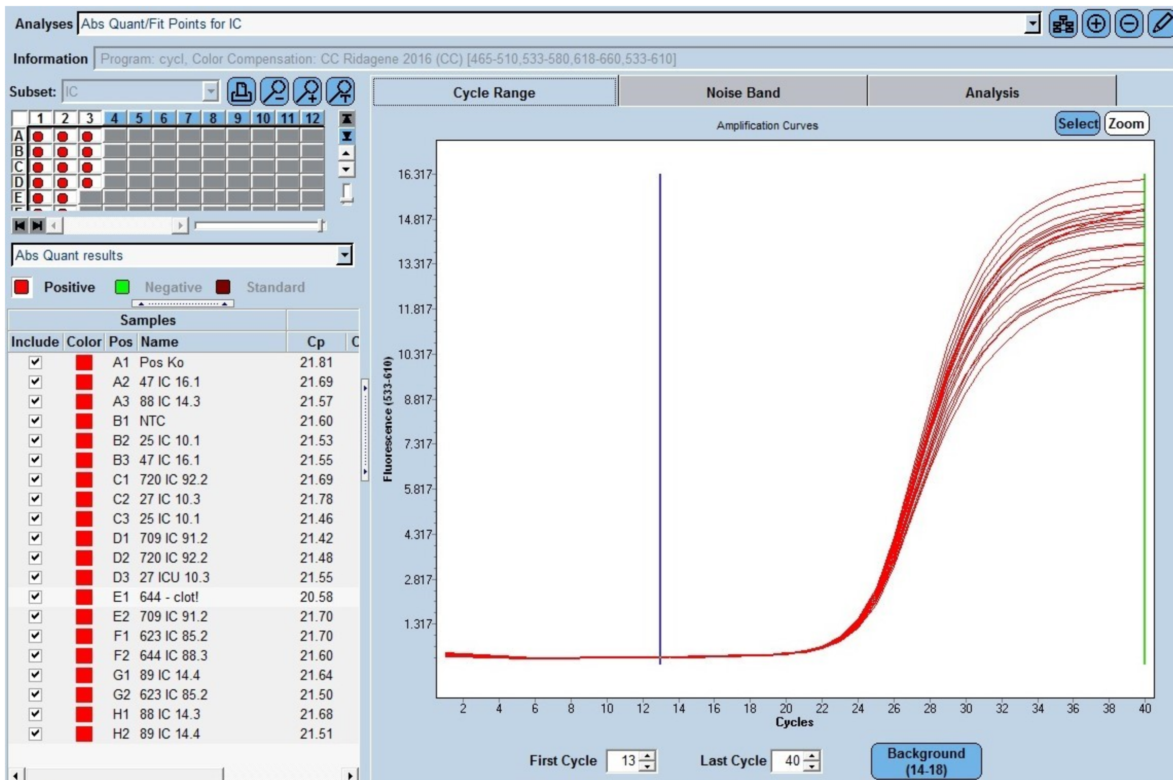

Figure 10. Lightcycler qPCR results for the internal extraction control with the *Cand*ID® kit (image source: Melina Jobstmann).

Table 8
Results obtained by the new *Cand*ID® and *Cand*ID PLUS® kits based on qPCR compared to those obtained by blood culture.

	qPCR pos.	qPCR neg.
Blood culture pos.	14	7
Blood culture neg.	1	30

4.3 Lab Flow Analysis

The turn-around time including hands-on time was estimated. Times required for the new *Cand*ID® and *Cand*ID PLUS® kits were identical. When 8 samples were extracted in parallel, the time required was 84 min for the fully automated extraction with the EMAG® platform and an additional hands-on time of 10 min. Preparation of the PCR mix and addition of eluted DNA took another 10 min. The time for amplification/detection on the LightCycler 480 II was 50 min. The average turn-around time was thus 154 min.

5. Discussion

Molecular assays for detection of *Candida spp.* DNA have become an important tool for diagnosis of candidemia. Potential advantages include same-day identification of *Candida spp.* and the possibility of relatively fast and continuous monitoring of persistence or resolution of infection. When using blood culture, the current gold standard for diagnosing invasive candidiasis, delays of up to 48 hours and possibly longer (in case information of specific species is required) must be expected for results (16). The new *Cand*ID® and *Cand*ID PLUS® kits are based on multiplex qPCR and have been designed for detection of three different *Candida spp.* each (*C. albicans, C. glabrata, C. parapsilosis* with the *Cand*ID® *and C. tropicalis, C. krusei, C. dubliniensis* with the *Cand*ID PLUS®). In this study, the analytical and clinical performance of both tests were evaluated, and results were compared to those obtained with blood culture.

The accuracy of the *Cand*ID® and *Cand*ID PLUS® kits was tested utilizing the Quality Control for Molecular Diagnostics (QCMD) 2018 *Candida spp*. EQA Programme. The panel consisted of 10 members including *C. albicans, C. auris, C. glabrata, C. krusei,* and vials without *Candida spp.* As expected, the kits did not detect fungal species other than *Candida spp.* and that sample containing *C. auris.* The negative panel member was correctly identified as negative. In one sample *C. parapsilosis* was found in addition to *C. albicans* whilst only *C. albicans* was stated in the panel. This sample was the only one containing synthetic bronchoalveolar lavage (BAL) instead of plasma as matrix. According to the QCMD department another false positive result for *C. parapsilosis* was detected by a participating laboratory in a synthetic BAL sample. However, on review of linked samples made from the same stock material there were no reports of *C. parapsilosis* in any of the other samples.

In the clinical study, 23 patients with candidemia and 31 patients without candidemia but bacteremia were included. Two EDTA whole blood samples obtained from patients with candidemia were found to be inhibited and were excluded from analysis. The performance of molecular assays may be affected by inhibitors. This problem exists especially with the wide range of non-blood specimens often used for detection of pathogens. Inhibitors exert influence on amplification through direct interaction with DNA or interference with DNA polymerases. Common specimen types known to contain inhibitors include blood,

sputum, urine, feces, and tissues. Additional sources of inhibitors may be materials and reagents that are exposed to samples during nucleic acid extraction. The optimal way to prevent inhibition is to remove possible inhibitors from being processed with the sample, dilution of the sample may also help (17). However, while viruses, bacteria, and even some parasites circulate in high numbers in body fluid, fungi often are not found in blood or body fluids at sufficient levels for simplified nucleic acid extraction protocols (18). Therefore, it may be preferable using increased specimen volumes for extraction of fungal DNA. To detect amplification failure due to inhibition, an internal quality control must be incorporated in every molecular test to exclude false-negative results. To ensure reliable test results, the internal control must be added to the sample before the start of the nucleic acid extraction procedure (17).

Fungi are problematic diagnostic targets due to their rigid cell wall that may be resistant to lysis. When using molecular methods for detection of fungal DNA, a sufficient nucleic extraction protocol should be employed. The protocol must be able to disrupt the cell wall, either by physical, chemical, and/or enzymatic methods. The specimen source also plays an important role in the extraction process. Whole blood does not seem to be the best substrate for fungal detection, different blood components may be better sources instead (18). The efficiency of extraction of fungal DNA from plasma or serum is higher than that from whole blood, and free DNA (in the serum and plasma fractions) is cleared more slowly than intracellular DNA, although it is not clear how they are protected from degradation. Whole blood contains both free and cell-associated fungal DNA but there is concern about loss of nucleic acids during centrifugation and removal of erythrocytes. However, there is currently no consensus on the optimal blood component for isolation of fungal DNA (19).

In patients with candidemia, only 14 of 21 samples showed a positive result with the new *Cand*ID® and *Cand*ID PLUS® kits. All *Candida spp.* in each of the 14 samples were correctly identified. *C. albicans* was isolated most frequently (10 times), followed by *C. glabrata* (3 times), *C. parapsilosis* (once), and *C. krusei* (once). The kits did not detect 5 samples containing *C. albicans* and 2 samples containing *C. glabrata.* In patients without candidemia 30 of 31 samples remained negative with the *Cand*ID assays. One sample containing *Peptostreptococci* was found to be positive for *C. parapsilosis* when using the *Cand*ID® kit. According to

our information about this patient, there was no clinical suspicion of candidiasis or candidemia. While a high specificity of the kits used in this study was found, a low sensitivity was observed which corresponds to a very recent multicenter study (16).

The new *Cand*ID® and *Cand*ID PLUS® kits could be completed within approximately 3 h. This is significantly faster in comparison to blood culture (up to 2 days or even longer).

Limitations of this study include the low number of patients and the fact that EDTA whole blood was used as substrate for fungal detection in patients with candidemia instead of plasma in patients with bacteremia. However, the study design was chosen according to real-world conditions in a high-throughput routine diagnostic laboratory. Furthermore, the samples had been stored for a rather longer time period. Larger studies with plasma samples of patients at risk for invasive candidiasis are suggested.

In conclusion, detection of clinically relevant *Candida spp*. with the *Cand*ID® and *Cand*ID PLUS® kits shows a comparable specificity but an inferior sensitivity when compared to blood culture. The major advantage of the new *Cand*ID® and *Cand*ID PLUS® kits is the significantly shorter time to diagnosis providing information about the Candida subspecies within less than 3 hours. With regard to the inferior sensitivity, only two-third of patients with candidiasis may profit by an early specific antifungal therapy. Further studies with increased sample size are strongly recommended.

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