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Fungal Infections Diagnosis – Past, Present and Future

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25 **Abstract**

26 Despite the scientific advances observed in the recent decades and the emergence of new
27 methodologies, the diagnosis of systemic fungal infections persists as a problematic issue. Fungal
28 cultivation, the standard method that allows a *proven* diagnosis, has numerous disadvantages, as
29 low sensitivity (only 50% of the patients present positive fungal cultures), and long fungal growth
30 time. These are factors that delay the patient's treatment and, consequently, lead to higher hospital
31 costs. To improve the accuracy and quickness of fungal infections diagnosis, several new
32 methodologies were implemented in clinical microbiology laboratories. Most of these methods are
33 independent of pathogen isolation, which means that the diagnosis goes from being
34 considered *proven* to *probable*. In spite of the advantage of being culture-independent, these
35 methods lack standardization. PCR-based methods are becoming commonly used, which has
36 earned them an important place in hospital laboratories. This can be perceived now, as PCR-based
37 methodologies have proved to be an essential tool fighting against the COVID-19 pandemic. This
38 review aims to go through the main steps of the diagnosis for systemic fungal infections, from
39 diagnostic classifications, through methodologies considered as "gold standard", to the molecular
40 methods currently used, and finally mentioning some of the more futuristic approaches.

41

42

43 **Keywords:** Fungal infections diagnosis; *proven* diagnosis; *probable* diagnosis; Gold standard
44 methodologies; PCR-based methodologies.

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1. Introduction

Throughout the years, the estimated number of fungal species around the world has gradually increased. In 2015, based on morphological, physiological, and molecular characteristics, this estimation reached 100,000 fungal species [1]. In the same year, the International Code of Nomenclature (ICN) reported that about 1000 to 1500 fungal species were being described and identified every year. In 2017, Hawksworth and co-workers [2] reported that the number of identified fungal species had reached 120,000, especially due to the progress of molecular genetics, representing, however, only 8% of the fungal species present on Earth. In fact, in 2017 the number of fungal species on Earth was estimated to be about 500,000 to ten million [1], increasing in 2019 to around 700,000 to 12 million species [3]. However, by 2020, the number of identified fungal species was only 140,000, according to Xu and co-workers [3]. Despite the high number of described fungal species, it is estimated that only 500 are associated with human, animal and plant infections, and that only 50 species are capable of infecting humans [1,4].

Recent ecological and climatic changes are leading to more frequent interactions between humans and wildlife. These changes are known to be responsible for the emergence of new pathogens, including fungal pathogens, since they allow the adaptation and proliferation of fungi to different ecological niches [4]. Nowadays, people benefit from the progression of medicine, providing an increase in the average life expectancy, as well as the improvement of treatments for various diseases. However, the development of medicine also increased the susceptibility of humans to fungal infections, especially due to the use of immunosuppressive therapies. These infections, whether caused by opportunistic fungi or by primary pathogens, are divided into superficial mycoses, allergic diseases and mycoses with an invasive character [2,4]. Fungal infections continue to be undervalued and underestimated both by the population and by public health organizations [5]. Diseases caused by protozoa, bacteria and viruses have been recognized

73 as a public health issue over the centuries, but the systemic fungal infections were only considered
74 as a relevant issue in the 80´s [5].

75 The Global Action Fund for Fungal Infections reported that, annually, more than 300
76 million people suffer from systemic fungal infections and, from these, about 1.5 million ends up
77 dying [6]. The most prevalent fungal pathogens in underdeveloped countries are *Cryptococcus* spp.
78 and *Pneumocystis* spp., generally associated with AIDS. Regarding developed countries, the most
79 frequently diagnosed invasive infections are those caused by *Candida* spp. and *Aspergillus* spp.
80 [7]. *Blastomyces*, *Histoplasma*, and *Coccidioides* are endemic fungi that can cause localized
81 infections, yet they can progress into systemic and have much more severe clinical implications in
82 high-risk patients. Disseminated histoplasmosis can be frequently encountered in
83 immunocompromised individuals and is mainly associated with AIDS patients. High mortality
84 rates of histoplasmosis in HIV-infected patients have been reported, ranging from 10 to 50% in
85 America [8]. However, pathogens such as *Malasezzia* spp. and *Trichosporon* spp. are also involved
86 in systemic infections but with a much less prevalence.

87 In 2021, there has been an increased concern related to the COVID-19 pandemic caused by
88 the SARS-CoV-2 virus. According to Sharma and co-workers [9], infection by SARS-CoV-2 leads
89 to a decrease in T cells, namely CD4+T and CD8+T, resulting in a debilitated immune system that
90 makes the patients more susceptible to contracting fungal infections. The fungal pathogen
91 commonly linked to post-COVID-19 infections is *Rhizopus arrhizus*, which belongs to the order
92 Mucorales, responsible for mucormycosis, and is frequently associated with the term “black fungi”
93 [10,11]. The association of COVID-19 to the “black fungi” is more evident in India. Despite this
94 recent association, the incidence of mucormycosis is related to certain predispositions, such as the
95 hygiene of the hospital environment (contamination of catheters and intravascular devices) and the

96 humidity of the country, which favours fungi reproduction [11]. Mucormycosis can have several
97 clinical manifestations - rhinocerebral, pulmonary, cutaneous, gastrointestinal and disseminated
98 [12] -, even though, however, post-COVID-19 infections are generally linked with rhinocerebral
99 and pulmonary conditions [11]. Ahmadikia and co-workers [13] compared the association of
100 mucormycosis with Influenza or with COVID-19 diseases. Mucormycosis combined with COVID-
101 19 infection, results in a more aggressive fungal infection, thus linked to higher mortality rates.
102 Those can be due to the overload of the health system, late diagnosis, and the weakened patient's
103 immune system that results in more critical fungal infections [13].

104 The COVID-19 pandemic might have increased the transmission of other nosocomial
105 fungal infections, like those caused by *Candida auris* that is considered a serious global health
106 threat, due to its high antifungal resistance and frequent transmission in hospital environments.
107 There are common risk factors for infections caused by SARS-CoV-2 and *C. auris*, such as
108 diabetes, contact with intubation systems, mechanical ventilation, and exposure to broad-spectrum
109 antibiotics. Therefore, *C. auris* outbreaks have been reported in COVID-19 intensive care units
110 [14–16]. Bayona and co-workers reported an increase of *C. auris* candidaemia cases during the
111 pandemic, in a Spanish hospital. The 28-day mortality rate for *C. auris* candidaemia was 57.1%
112 until March 2021 [15].

113 Several actions have been proposed to reduce deaths related with systemic fungal infections
114 [17], such as the prophylactic administration of antifungal after evaluating the patients' clinical
115 symptoms and risk factors associated with a fungal infection, but also the efforts to reach a
116 definitive diagnosis as fast as possible. If these actions were followed, it was estimated that by
117 2020, deaths caused by meningitis triggered after infection by *Cryptococcus*, would have been
118 reduced from 180,000 to 70,000, annually. In addition, deaths caused by *Pneumocystis* infections

119 would have declined from 400,000 to 162,500, histoplasmosis-related deaths would have decreased
120 by 60%, and deaths by aspergillosis-related pneumonia could be decreased from 56,000 to 33,500.
121 If these actions were followed, after 5 years, one million lives would have been saved [17].
122 To prevent pandemics, it seems clear that public health organizations need to consider
123 systemic fungal infections as contemporary and a real problem, as has been observed previously in
124 other models of infectious diseases. In addition, since these infections are less known and caused
125 by less-studied pathogens, they represent a greater risk to public health, and should concentrate
126 higher attention [18].
127

128 **2. Fungal Infections Diagnosis**

129 The European Organization for Research and Treatment of Cancer/Invasive Fungal
130 Infections Cooperative Group (EORTC) and the National Institute of Allergy and Infectious
131 Diseases Mycoses Study Group (MSG) established definitions incorporating the parameters of the
132 diagnosis of fungal infections at a clinical level. Those have been extremely useful for researchers
133 conducting epidemiologic studies, diagnostic assays, and antifungals clinical trials. The 3 levels of
134 classification of Invasive Fungal Infection (IFI) diagnosis are *proven*, *probable*, and *possible* [19–
135 21]. These definitions, established in 2002, only covered the diagnosis of fungal infections related
136 to immunocompromised, oncological, and hematopoietic stem cell transplant patients [19].
137 The *proven* diagnosis requires the detection of the pathogenic fungi through
138 histopathological or culture methods from sterile sites [20,21]. For the *probable* and *possible*
139 diagnosis to be attributed, three variables have to be analyzed: (i) the host factor - is related to the
140 patient's risk of contracting a fungal infection, thus several parameters are evaluated, such as recent
141 history of neutropenia, receipt of an allogeneic stem cell transplant, prolonged use of
142 corticosteroids, immunosuppressants therapy, and inherent immunodeficiency; (ii) clinical signs

143 and symptoms related to the fungal infection, so some clinical manifestations are taken into
144 consideration as tracheobronchitis, sinonasal infection and central nervous system infection; (iii)
145 mycological evidence, accompanied by the positive result of a diagnostic test, either conventional
146 or molecular [20,21].

147 Thus, in 2008, these definitions were updated and redefined and the *possible* has been
148 attributed to cases where the fungal infection is highly probable but mycological evidence is
149 lacking [20]. In 2019, a new revision and updating of the consensus definitions established that the
150 *proven* IFI classification could be applied to any patient (immunocompromised or not) and that the
151 *probable* and *possible* classifications were only projected for immunocompromised patients [21].
152 The *probable* diagnosis requires a host factor, a clinical feature, and mycologic evidence.
153 Excluding these factors, endemic mycoses cases without mycological evidence are considered a
154 *possible* IFI [21].

155 Pathogenic fungi detection can be obtained through several approaches, from traditional
156 fungal cultures to molecular Polymerase Chain Reaction (PCR)-based methods [20,21]. A variety
157 of tests are available and, preferably, more than one type of test should be applied to the patient if
158 an invasive fungal infection is suspected. In Table 1 we review the advantages and disadvantages
159 of each test. By testing the patient with two different tests, it leads to a more effective and robust
160 diagnosis. Since host factors, clinical signs and symptoms are not under the scope of this review,
161 we will focus on the mycological evidence. For further analysis on the previous parameters some
162 reviews are available. Zhang and co-workers [22] analyzed the clinical characteristics of 145 cases
163 of invasive fungal infections. Webb and colleagues [23], analyzed the incidence, clinical features
164 and outcomes of invasive fungal infections in the US health care network, according to 3374
165 episodes in 3154 patients.

166 The diagnosis of a fungal infection is a lengthy process, especially due to the symptomatic
167 similarities between bacterial and fungal infections. The time to reach a differential diagnosis of
168 the patient is long, and delaying the patient's diagnosis will consequently delay their treatment
169 [5,24]. However, molecular methodologies allowed to significantly reduce the turn-around time,
170 by introducing methodologies that permit to obtain more specific, efficient, fast, and accurate
171 results. This means that the overall diagnosis process is faster, which allows an adequate and timely
172 delineation of the therapeutic plan, increasing the survival rate. This also leads to a reduction of
173 people admitted to intensive care units, which can yield the hospital approximately $30,000 per
174 patient [25].
175 The correct identification of the pathogenic fungi at the species level is fundamental to
176 better understand the epidemiology of the infection. In Figure 1 a workflow is provided, reviewing
177 the diagnosis of a systemic fungal infection. Several techniques are assessed (further detailed in
178 this review and compared in Table 1) in terms of time consumption, specificity, sensitivity,
179 automation, among others. With this review we plan to make available a quick chart to optimally
180 choose among the existing molecular methods. Only with the proper method it will be possible to
181 achieve a specific treatment, which is crucial for the patient’s survival.
182
183 **3. *Proven* diagnosis**
184 **3.1. Workflow of clinical diagnosis**
185 In fungal infections diagnosis, cultivation in appropriate media, direct microscopy, and
186 histopathology are still the techniques routinely used to obtain a definitive diagnosis. Even when
187 replaced by other more modern techniques, conventional methodologies continue to be employed
188 as comparison and for confirmation [26].
189

3.1.1. Fungal cultures

If systemic fungal infection is suspected, the host factors and the clinical signs and symptoms of the patient are firstly analysed. If all factors point to an invasive fungal infection, the start-off is to try to isolate the pathogenic fungi. For this, sterile liquids, such as blood, urine and cerebrospinal fluid are collected. When the growth of the microorganism in cultures is positive, using these sterile fluids, the diagnosis is direct. On the other hand, when using non-sterile fluids, like bronchoalveolar fluid, commensalism needs to be considered. Despite cultivation being declared as gold standard methodologies for diagnosis and identification of the fungal species, this method is associated with low sensitivity. The overall sensitivity for yeasts is about 50 to 60%, and for molds 30 to 68% [26,27].

Regarding invasive candidiasis, the golden standard approach to diagnosis is blood culture. Ericson and colleagues [28] evaluated the effectiveness of several commercially available blood culture vials at detecting *Candida* species. In this study the BacT/Alert FA vials were able to detect 144 of 179 samples (80.45%), proving to be the most efficient when compared to others (Bactec Mycosis IC/F and BacT/Alert FN). Another important factor was the fact that it was shown that anaerobic vials (BacT/Alert FN) were not successful in identifying the *Candida* growth (8 samples were detected out of 179) [28]. It was also observed that the vast majority of the blood culture vials take about 14 to 72 hours to grow a significant amount of *Candida* cells [29].

Candiduria (presence of *Candida* species in the urinary tract) may also often be associated with the presence of *Candida* spp. in the bloodstream (candidemia). According to the literature, candidemia is associated with 40 to 68% of the cases of candiduria [30,31]. Therefore, in case of suspected candidemia, an alternative workflow could also be to use urine cultures, where the most commonly used media is a chromogenic clear media (Oxoid Ltd, Basingstoke, UK) [32].

213 Regarding cerebrospinal fluid samples cultivation to detect fungal species, such as
214 *Cryptococcus* and *Cladosporium*, the most appropriate media for pathogens growth are a
215 Sabouraud 4% dextrose agar and sheep blood agar plates [33]. These pathogens usually take about
216 3 to 7 days to grow, and the colonies are cream-colored, having a mucoid appearance [27].

217 In the case of molds, obtaining the clinical isolate through culture media is even more
218 complicated, since the sensitivity associated is very low (30 to 68%) [27]. Another drawback is
219 that these type of pathogens requires a long time to grow, explicitly up to two weeks, and by then
220 when molds grow there is always the hypothesis of external contamination [26]. Guegan and
221 colleagues [34] identified *Aspergillus* spp. from 413 samples, from 387 immunocompromised
222 patients. The detection of *Aspergillus* spp. from bronchoalveolar fluid culture was much lower
223 (47%) when compared to other non-cultivation methods like galactomannan assay (87%), and
224 PCR-based assay (60 to 75%) [34]. Tarrand and co-workers [35] demonstrated that incubation of
225 the cultures at 35ºC provided higher sensitivity (a 31% increase) when compared to incubation at
226 25ºC. This is explained by the fact that at 35ºC there is a greater similarity between the incubation
227 environment and the environment within the host [35].

228 Fungal cultures represent a widely used methodology that enables microorganisms'
229 detection and antifungal susceptibility testing. However, most standard culture media such as
230 Sabouraud dextrose and malt extract agar [36] only provide information about the presence/absence
231 of microorganisms, and so, additional methods are needed to perform species identification.

232

233 **3.1.2. Direct Microscopy and histopathology**

234 Direct microscopy is applied to analyze the morphological structures of the fungi in culture
235 after their growth, or in a portion of infected biopsy tissue or fluid. This allows to evaluate whether
236 the infection is triggered by a septate mold (such as *Aspergillus* spp.), a non-septate mold (for

237 example *Mucorales*), or a yeast (for example a *Candida* spp.) [37]. Throughout the visualization
238 of the fungi's appearance in the tissue section and identification of specific morphological patterns,
239 it is possible to differentiate between different histopathological diagnoses associated with invasive
240 fungal infections. However, the visualization of those structures alone does not provide a specific
241 identification since the analyzed structures are similar in various fungal species [38]. Nevertheless,
242 histopathology is very useful to avoid false positive/negative results from the fungal culture or
243 cases of uncultivable fungi, respectively. Additionally, it is very important to assess tissue invasion
244 to understand the significance of the isolate (pathogenic fungus / normal microbiota /
245 environmental contamination). Visualization of fungal structures by histopathology and direct
246 microscopy techniques can respectively be improved, through the use of stains, such as Gomori's
247 methenamine silver or the periodic acid–Schiff reaction [38], and fluorescent brighteners, such as
248 Calcofluor white [36].

249 These conventional techniques together remain as golden standard methods for stating the
250 diagnosis of fungal infections due to several advantages as they allow to (i) evaluate antifungals
251 resistance, (ii) visualize the fungal structures, and (iii) confirm results obtained by biochemical and
252 molecular methodologies. However, these diagnostic approaches have inherent limitations,
253 according to the evidence collected in this review, being time-consuming and frequently
254 accompanied by incorrect species identification. Moreover, their lack of sensitivity and the
255 relatively slow achievement of the results often lead to delayed clinical decisions and therapeutic
256 actions, which are important determinants for the infection outcome of the patient.

257

258 **3.2. Workflow after pathogen isolation**

259 Following the growth of the pathogenic fungi in an appropriate culture media, the
260 information that is obtained is simply related to the presence or absence of the pathogen. Therefore,

261 in order to be able to identify the fungal species behind the infection, there are complementary
262 methodologies used to achieve a specific identification, leading to a better therapeutic plan.
263
264 **3.2.1. Chromogenic media**
265 Chromogenic media has been widely used in clinical microbiology to detect and identify
266 either bacterial or fungal pathogens [39], being used for *Candida* identification since 1994.
267 Considering the unspecific clinical scenarios, the detection of the presence or absence of a fungal
268 pathogen is frequently insufficient, thus chromogenic media can be used to overcome this
269 limitation [40]. They allow the growth of a specific microorganism, and its identification is based
270 on reactions that occur in the culture medium, since the culture has a substrate enzyme linked to a
271 chromogen (color reaction), or linked to a fluorogen (light reaction), or even a combination of both
272 [40]. These culture media are suitable for non-sterile samples as they stimulate the growth of a
273 specific genus, inhibiting the growth of other microorganisms [26]. CHROMagar® *Candida* (BD
274 Difco), *Candida*® ID2 (bioMerieux), Hicrome® *Candida* (HiMedia), CandiSelect™ 4 (CS4) and
275 Brilliance™ *Candida* Agar (BCA) are examples of commercially available media for *Candida*
276 species identification [40].
277
278 **3.2.2. Phenotypic biochemical identification systems**
279 Several phenotypic systems have also been developed and are commercially available.
280 These systems are most suitable for yeast species as for instance the manual API® 20C AUX and
281 the automated VITEK® 2 (bioMérieux, France). This sort of biochemical kits have been extensively
282 reviewed and evaluated throughout the years [41,42], being commonly used in routine mycological
283 diagnosis to identify and assess antifungal susceptibility of fungal species isolated from clinical

284 samples. Therefore, before performing these methods, it is necessary to obtain a pure culture of the
285 pathogen [42].

286 A recent study [43] aimed to compare the performance of the API® 20C yeast identification
287 system with other molecular methods. The results showed that API® system properly identified
288 97.26% of the most common *Candida* species. However, this system was not equally suitable for
289 rare yeast species. Furthermore, it was described as the least accurate and least economic technique
290 discussed. The VITEK® 2 automated identification system can also appropriately identify most
291 clinically relevant *Candida* species. Ambaraghassi *et al*. reported that the VITEK® 2 had limited
292 ability to distinguish between *C. auris* and closely related species, only correctly identifying about
293 52% of the *C. auris* [44].

294

295 **3.2.3. Matrix-Assisted Laser Desorption/Ionization – Time Of Flight (MALDI-TOF)**

296 In recent decades, mass spectrometry-based methodologies gained popularity in
297 microbiology laboratories because they provide fast identification at low costs, with easy
298 accessibility and great applicability to several microorganisms. Regarding the identification of
299 fungal species, the variation of mass spectrometry most widely used is matrix-assisted laser
300 desorption/ionization (MALDI-TOF), which is based on the identification of fingerprints of
301 extracted proteins, mainly ribosomal and membrane proteins. The proteic profile obtained for each
302 isolate is compared with universal profile databases, enabling identification at the species and
303 genus level [26,27].

304 Becker and colleagues [45] identified 290 fungal isolates, at species level, including filamentous
305 fungi and yeasts, belonging to 69 different species, through conventional culture methodologies
306 and by MALDI-TOF. In the study, the identifications were confirmed by DNA sequencing of the
307 isolates, and the results obtained by MALDI-TOF, and cultivation were compared. MALDI-TOF

308 was able to correctly identify 89% of the species, while conventional cultures only achieved 69%
309 of correct identifications [45]. Lau and co-workers [46] developed a spectra database according to
310 249 fungal isolates, which was used to identify 421 clinical isolates, through MALDI-TOF. This
311 database was able to correctly identify about 90% of the isolates when compared with the results
312 obtained from DNA sequencing. Several studies have been carried out to analyze the performance
313 of MALDI-TOF methodology in identifying fungal species, and the results are promising.
314 Therefore, this methodology has the potential to replace conventional methodologies for the
315 identification of pathogenic fungi [26,27].

316

317 **3.2.4. Fluorescence *in situ* hybridization (FISH)**

318 In hospital microbiology laboratories, FISH is routinely used to detect pathogenic
319 microorganisms from positive blood cultures. This technique can be used individually or as a
320 complement to other techniques [47]. FISH is based on fluorescent probes that bind to a specific
321 sequence of the microorganism's genome, and in the case of fungal species to the 18S region of
322 the rDNA. When the probe binds to its target, fluorescence can be visualized using fluorescence
323 microscopy [27,48]. The most used probes for this assay are DNA-based FISH probes, however
324 Peptide Nucleic Acid (PNA)-based FISH probes can also be used. PNA-based FISH probes are
325 appearing more frequently on the market since they have a neutral backbone that minimizes
326 interference in microscopic visualization, however they are more expensive [48].

327 Silva and co-workers [47] compared the potential of the FISH methodology when
328 identifying fungal species with that presented by traditional cultures and microscopy, using 30
329 blood cultures. Of the 30 blood cultures, 14 ended up presenting fungal growth which were later
330 identified through the two different methodologies. The identification of the pathogen was in
331 agreement between the FISH methodology and the culture and microscopy analysis. However,

332 culture and microscopy identification methods need specialized clinics to carry out the
333 identification and are time-consuming (3 to 10 days). In contrast, the FISH methodology presented
334 the same results within 5 hours [47].

335 PNA-FISH® was the first platform based on this method to be commercialized and applied
336 in the hospital routine. This kit uses PNA-based FISH probes that detect several sequences of
337 pathogenic microorganisms, such *as Staphylococcus aureus*, *Enterococcus* spp., gram-negative
338 bacteria, and *Candida* spp. [48]. The disadvantages associated with the PNA-FISH® platform are
339 especially the limit of detection presented by this technique, and the reduced number of PNA
340 probes available in the market. In addition, the most crucial limitation is the need of positive blood
341 cultures in order for the methodology to be used. However, this platform is capable of displaying
342 results within two hours, with sensitivity and specificity of 97 and 100%, respectively [48].

343 Klingspor and colleagues [49] evaluated the clinical use of the Yeast Traffic Light PNA
344 FISH (AdvanDx, Inc., Woburn, MA) (YTL PNA FISH), when identifying *Candida* spp. This kit
345 is based on a FISH assay and differentiates 5 *Candida* spp. according to their susceptibility to
346 fluconazole, by visualizing 3 different colors. Green stands for susceptible to fluconazole treatment
347 (*C. albicans* and *C. parapsilosis*), yellow means that a higher dose of fluconazole must be
348 administrated (*C. tropicalis*), and red represents a natural resistance to fluconazole (*C. krusei* and
349 *C. glabrata*). Of 137 patients positive blood cultures included in the study without antifungal
350 treatment, the YTL PNA FISH was able to correctly target the treatment of 132 patients (96.4%),
351 and distinguish between bacteria and yeasts in a concomitant growth (95.8%) [49].

352

353 **3.2.5. PCR-based methodologies**

354 Several PCR-based methodologies are available to fulfil the objective of identify the
355 pathogenic fungi, after obtaining the fungal isolate. FilmArray® is a fully automated platform that

356 incorporates steps from sample preparation, PCR amplification and detection/identification of the
357 pathogen [48,50,51]. This method allows to detect, with success, 19 species of bacteria, 5 *Candida*
358 species, and some resistance genes through positive blood cultures. Moreover, these identifications
359 are associated with high values of sensitivity and specificity (96% and 99%, respectively) [50].
360 Additionally, it is effective in cases of mixed infection. Despite providing results in one hour, only
361 one sample at a time can be used. However, with the introduction of FilmArray® Torch, it is
362 currently possible to run 2 to 12 samples at a time [48].

363 Sepsis Flow Chip is a new platform that combines real-time PCR with a reverse dot blot
364 hybridization for the detection of the most common pathogens in systemic infections, through
365 positive blood cultures [48,52]. This methodology is able to identify 36 species of bacteria, several
366 *Candida* species, and more than 20 resistance genes, in 3 hours. In its validation and verification
367 trial, this platform obtained high values of sensitivity and specificity regarding *Candida* species:
368 93.3 and 100%, respectively [52]. It also showed excellent results when identifying cultures with
369 more than one pathogen [52].

370 ePlex® is a fully automated platform, incorporating all the necessary steps for the analysis
371 of positive blood cultures. It has a sample preparation system, followed by a multiplex PCR
372 amplification system, and finally the amplicon analysis through electrochemical examination
373 [48,53,54]. It has several panels that allow the detection of various pathogens such as gram-
374 negative and gram-positive bacteria, and fungal species, from blood cultures. Regarding the
375 identification of fungal pathogens from blood cultures, the ePlex® system was able to correctly
376 identify 100% of the species [53,54].

377

378 **4. *Probable* diagnosis**

379 When no detection of the pathogenic fungi through histopathological or culture methods
380 from sterile sites is possible, but only detection of traces of the pathogen, a *probable* diagnosis is
381 attributed. Serological, molecular and other more recent techniques are available to collect
382 evidence of the presence of the pathogenic fungi. Some of these methodologies can also be used
383 after a positive blood culture for species identification.

384

385 **4.1. Serological methodologies**

386 The development of laboratory markers and the launching of antigen testing have improved
387 the diagnosis of invasive fungal infections regarding quickness and efficiency. Fungal antigens,
388 metabolites, or antibodies produced by the host's immune system can be detected in several serum
389 samples, but also urine and bronchoalveolar fluid [38].

390

391 **4.1.1. β-(1,3)-D-glucan assay**

392 β-(1,3)-D-glucan is a polysaccharide present in the cell wall of several fungi, and its
393 detection can indicate a variety of infections, from invasive candidiasis, to invasive aspergillosis
394 and also infections caused by *Pneumocystis jirovecii* [27]. The Fungitell® assay is one of the best
395 commercialized tests, presenting high sensitivity and specificity values (79% and 89%,
396 respectively) [55]. Wako β-glucan test is another commercially available assay which presents high
397 values of sensitivity and specificity in measuring the β-(1,3)-D-glucan biomarker. It presents a
398 variety of sensitivity and specificity values depending on the type of fungal pathogen. Regarding
399 invasive aspergillosis, this assay allows to obtain, for example, 80% and 97.3% of sensitivity and
400 specificity, respectively [56]. For candidiasis, these values are even higher - 98.7% and 97.3%,
401 respectively -, and for *Pneumocystis* spp. are 94.1% and 97.3%, respectively [56]. Racil and co-
402 workers [57] aimed to evaluate the efficiency of β-glucan assay in patients with haematological

403 malignancies, however, a high number of false-positives results were observed. Although they
404 were not able to confirm any of them, the authors tried to formulate several hypotheses. The first
405 one was related to contamination of the catheters with fungal DNA and the other was associated
406 with the sensitivity of the assay, being difficult to interpret the results and to differentiate an active
407 infection from colonization [57]. Mennink-Kersten *et al.* [58], reported that bacteria such as
408 *Alcaligenes faecalis*, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* showed β-(1,3)-D-
409 glucan reactivity with the Fungitell® assay, which can also provide false-positive results.
410 According to Hammarström and colleagues [59], patients receiving treatment with pegylated
411 asparaginase and ICU patients treated with plasma, albumin, or coagulation factors, showed
412 elevated levels of β-(1,3)-D-glucan, being more likely to test positive for the β-glucan assays.
413

414 **4.1.2. *Candida albicans* Germ Tube Antibody (CAGTA) assay**

415 Regarding *Candida* species, a broad range of serologic tests are also available, as for
416 instance the CAGTA assay and Mannan detection, which is the major *Candida* cell wall antigen,
417 significantly associated with systemic candidiasis [27]. *C. albicans* germ tube antibody assay
418 (CAGTA) is a test that aims to detect specific antibodies, produced to attack *C. albicans'* germ
419 tubes, achieved through indirect immunofluorescence [60]. In order to assess the effectiveness of
420 this assay, Zaragova and co-workers [61] used it in patients diagnosed with *possible* systemic
421 fungal infection. This study concluded that patients who were tested with the CAGTA assay and
422 treated accordingly, showed lower mortality rates when compared to those who did not. This was
423 able to prove the efficiency of the CAGTA methodology, since patients who tested positive for the
424 assay were treated with a generic antifungal, and survived [61].
425

426 **4.1.3. Galactomannan (GM) assay**

427 For invasive aspergillosis, GM is the main cell wall antigen detected in serum, in
428 bronchoalveolar fluid or in the cerebrospinal fluid. The sensitivity of GM assay is higher when
429 bronchoalveolar fluid is used (90%), followed by serum (71%) [27]. The commercially available
430 assay to detect GM, the ELISA Plateia *Aspergillus* assay™, is the most frequently used in the
431 clinical context to diagnose invasive aspergillosis. Nonetheless, this assay has a higher sensitivity
432 to *Aspergillus* non-*fumigatu*s species, which turns out to be a drawback, because *A. fumigatus* is
433 the prevalent pathogen in invasive aspergillosis [27]. Despite GM being present in the cell wall of
434 *Histoplasma capsulatum* and *Fusarium* spp., this antigen detection assay is mentioned as an
435 *Aspergillu*s-specific methodology [62–64]. In a study piloted by Tortorano and colleagues [63],
436 several *in vitro* and *in vivo* experiments were able to demonstrate the cross-reactivity between
437 *Fusarium* spp. antigens with the Plateia *Aspergillus* assay. This result turns out to be a disadvantage
438 for the specificity of the kit since it was described as being *Aspergillus* spp. specific. However this
439 kit can be a useful tool for the diagnosis of infections caused by *Fusarium* spp., since there is no
440 antigen test for this pathogenic species [63]. Also, despite being poorly studied for *Histoplasma*
441 *capsulatum*, this method turns out to be useful for the diagnosis of histoplasmosis, since this fungal
442 species takes about 4 weeks to grow in culture [62,64].

443

444 **4.1.4. Lateral-flow devices**

445 Other serological assays are also commonly applied for the diagnosis of *probable*
446 infections, such as the lateral-flow devices to detect galactofuranosis antibodies in serum or
447 bronchoalveolar fluid. This assay is specific for *A. fumigatus* and shows a specificity of 100% and
448 a sensitivity of 48 to 100%, which shows best results when comparing this assay with the 1,3-β-D-
449 glucan assay [65,66]. Due to its easy performance, it can be applied to point-of-care (POC) testing,
450 obtaining the result in 15 minutes [67].

4.2. Nucleic acid molecular methodologies

Several studies showed that rapid identification of the infectious agent leads to an appropriate therapeutic plan, which results in a lower mortality rate [26,48]. Since 1990, thousands of studies referring to the diagnosis of fungal infections through molecular methodologies have been published. However, the use of these techniques in hospital settings has been hampered by the lack of standardization and accreditation [24]. Molecular methodologies have also evolved to be totally independent of the growth of the microorganism in blood culture.

The majority of molecular methodologies used in clinical context were first developed in research laboratories and entitled "research use only" (RUOs) [24]. In order to reach bioindustry and clinical laboratories, those methodologies must undergo a rigorous process of verification and validation controlled by several entities [68,69]. Throughout the verification process, the new method is defined, characterized, and compared with the gold standard methodology, considering the disease or condition it aims to diagnose. This process allows the research center to evaluate the limitations, risks of error, and the likelihood of causing changes in the interpretation of the test results or treatment decisions [24,68,69]. The validation process incorporates the methodology quality control, that is assessed during the time it is commercially available, to guarantee that it works the way it was intended [24,68,69]. Regarding the validation and verification of molecular methodologies for invasive fungal infections, there is a special concern since gold standard techniques show inconsistent results, associated with lower rates of specificity and sensitivity. So, comparing a new molecular methodology with the gold standard, as for example cultivation in appropriate media, may result in the conclusion that the new methodology is not suitable [24,27].

4.2.1. PCR-based methods

475 In clinical terms, PCR-based methodologies are commonly associated with the direct use
476 of samples from sterile sites such as whole blood and cerebrospinal fluid, or from nonsterile sites
477 like bronchoalveolar lavage, to detect fungal DNA (Fig. 1).

478 Nucleic acid amplification-based methodologies consist of enzymatic processes in which
479 one or more enzymes can synthesize copies of target sequences. That is achieved through a pair of
480 primers, which specifically bind to the target sequence, resulting in the amplification of that
481 sequence. The biggest drawback of these methods is contamination, which may lead to the
482 amplification of unwanted sequences [24]. Polymerase chain reaction was the first nucleic acid
483 amplification methodology being developed and remains the most used in both clinical context and
484 scientific research. It has evolved and became more sophisticated, with novel variants of the
485 technique, specifically conventional PCR, reverse transcriptase-PCR, nested PCR, and real-time
486 PCR. Regarding fungal pathogens detection, conventional PCR and real-time PCR are the most
487 widely used, presenting high sensitivity, easy handling, and allowing identification of the pathogen
488 in a short time (Fig. 1) [24,27,70].

489 Lately, the scientific community has been making efforts to overcome and minimize the
490 biggest challenges of PCR methodologies. For instance, the fungal burden associated with invasive
491 fungal infections is very close to the limit of detection of PCR methodologies, so DNA extraction
492 is a crucial step in the diagnosis [24,27]. Fungi, especially molds, have a rigid cell wall, which
493 poses an obstacle for fungal DNA isolation and detection. Another complication is the
494 omnipresence of fungi which increases the risk of contamination and false-positive results. Also,
495 human DNA and other components in clinical samples can inhibit or interfere with the PCR
496 reaction [27].

497 In clinical contexts, the use of conventional PCR to detect and identify pathogenic
498 microorganisms is linked to an extra step for PCR product analysis, which increases the risk of

499 contamination by external factors. Another disadvantage is the lack of quantification of the PCR
500 products, precluding the differentiation between commensal colonization and active infection
501 [26,27].

502 Regarding conventional PCR, amplicon analysis is frequently done through (i) sequencing
503 - amplified products are sequenced to perform pathogenic fungi identification at species or genus
504 level [24,71]; (ii) FISH - this methodology is used for amplicon analysis by adding specific
505 fluorescent DNA probes to the PCR products, and the binding can be visualized by fluorescent
506 microscopy [47]; (iii) restriction fragment length polymorphism (RFLP) - PCR-RFLP is described
507 as a useful tool that allows the rapid differentiation of several microorganisms, specifically fungal
508 species, using restriction enzymes. To differentiate fungal species, MSP1 is frequently used.
509 Species differentiation is based on the pattern observed and on the size of the PCR product after
510 digestion [26,72]; and (iv) capillary electrophoresis - the PCR fragments are analyzed according to
511 their size. Products with close size can be distinguished by introducing different fluorescent labels
512 in one of the primers [65].

513 Real-time PCR enables the monitorization and quantification of the DNA over time,
514 implying that the data is collected and visualized as the reaction proceeds. This methodology occurs
515 entirely in a closed system, with no transfer of samples, no addition of reagents, or electrophoresis
516 [24,27,73]. Several fluorescent reporters are used to monitor real-time PCR, being divided into
517 intercalation and hybridization dyes [24,74]. Intercalation dyes become fluorescent in the presence
518 of dsDNA. The amount of DNA present in the sample is proportionally related to the fluorescence
519 observed on the monitor. However, intercalation dyes, like SYBR Green and EvaGreen, bind to
520 any dsDNA, which is also the case of primer-dimers or contaminating DNA. Nevertheless, these
521 dyes are low-cost and prevent the need to resort to probe design [24,74–76]. On the other hand, for
522 more rigorous monitoring of the amplification in real-time, hybridization dyes should be used.

523 Hybridization dyes are highly specific since they combine the specificities of the primer and the
524 probe, and can also be used in a multiplex system if their design is suitable [74–76]: (i) TaqMan
525 probes are related to the phenomenon of fluorescence resonance energy transfer (FRET) between
526 a reporter and a quencher. They are able to bind to the target sequence, and when DNA polymerase
527 begins to synthesize a new sequence, the probe is cleaved. Due to a greater physical separation
528 between the reporter and quencher, there is fluorescence emission by the reporter that is detected
529 by the device [24,71]; (ii) Molecular beacons are based on displaceable assay and are also
530 incorporated with a reporter and quencher for monitoring fluorescence. They are closed system
531 probes, in which the sequence of its loop is complementary to the target sequence [24,75]; (iii)
532 Scorpion primers are probes incorporated directly into the primers. Therefore, scorpion primers are
533 composed by the primers for the target region, and the probe is also a closed system where the loop
534 has a sequence complementary to the target sequence, similar to molecular beacons [24,75].

535 Nonetheless, hybridization dyes can be used in multiplex situations, although they depend
536 on the efficiency of the equipment [24]. In this case, each probe would be associated with the
537 detection of a specific microorganism, with a specific fluorescence, even though the equipment
538 would have to be able to detect several fluorescences simultaneously [24,77]. Still, hybridization
539 dyes can be used in a multiplex methodology where the equipment is capable of detecting only one
540 fluorescence [24]. In this case, each probe would be linked to the detection of a specific
541 microorganism, however with only one fluorescence [74]. Thus, an extra analysis of the products
542 would have to be carried out, through melting curve analysis [24,77].

543 Melting curve analysis (MCA) is a methodology with high sensitivity values, based on the
544 association of different amplicons to different melting temperatures. Those melting temperatures
545 are mainly determined by the guanine and cytosine content, but also by the size of the amplicon
546 [24,77]. MCA usually accompanies the use of TaqMan probes or SYBR Green. TaqMan probes

547 are related to better results since they specifically bind to the target region, and only those
548 amplicons will be analyzed via MCA [77]. Concerning SYBR Green, since it binds non-
549 specifically to all the dsDNA present in the sample, all amplicons will be analyzed through MCA,
550 by monitoring the decrease in fluorescence, and for this reason, it requires a more careful analysis
551 [24,71,77]. Xiao and colleagues [78] developed a real-time PCR methodology capable of
552 identifying 28 pathogens, including bacterial and fungal species. This assay used TaqMan probes
553 to ensure a more specific target sequence amplification, and the PCR products were analyzed via
554 melting curve analysis. The real-time PCR assay was used to identify 269 cases of positive blood
555 cultures, in which the pathogens present in the cultures would have already been previously
556 identified through MALDI-TOF. Real-time PCR assay showed great potential in identifying the
557 28 pathogens that it was designed to, presenting a sensitivity of 99.2%, a specificity of 100%, and
558 99.9% agreement with fungal cultures. However, in clinical practice, it presented an overall
559 sensitivity of 88.8%, since real-time PCR results remained negative for cases where the
560 methodology was not designed to identify a specific pathogen [78].

561 PCR methodologies can be utilized to detect all fungi (Panfungal PCR) by using universal
562 primers for highly conserved regions of the fungal genome, thus being possible to detect any fungal
563 DNA in a sample, even the rarest species. The specific identification of the fungal pathogen can be
564 achieved by sequencing, which increases the risks of contamination, or performing a specific PCR
565 [71,79]. There is a benefit associated with the combined use of panfungal and specific PCRs. In
566 this case, the medical procedure for diagnosing a systemic fungal infection, if a pathogenic fungus
567 is suspected, is a specific *Candida* or *Aspergillus* PCR test. In case of a negative result, a panfungal
568 assay should be performed to abolish the hypothesis of fungal infection, and then direct the
569 diagnosis to a bacterial infection [24,79]. In a study conducted by Camp and co-workers [79], the
570 sensitivity and specificity values of Fungi Assay (real-time panfungal PCR) were compared with

571 those presented by the “gold standard” methodologies, in particular fungal cultures. Regarding
572 Fungi Assay, if an amplification curve was observed, the PCR products were sequenced for specific
573 identification of the pathogen. On the other hand, when culture growth was verified, microscopy
574 and MALDI-TOF were used for specific identification. For this study, 265 clinical samples were
575 used, and the results were in agreement between Fungi Assay and fungal cultures in 55.1% of the
576 cases. However, in 5 samples, the Fungi Assay was able to detect a fungal pathogen while fungal
577 cultures remained negative. It was also claimed that this assay performed better when using
578 samples from sterile sites [79]. This study was innovative, and Fungi Assay was found to have a
579 great potential of diagnosis in cases where there was strong evidence of fungal infection. This assay
580 provided accurate and faster results when compared to fungal cultures. However, as it is a
581 methodology based on panfungal primers, it is normal that it has lower sensitivity than those that
582 use specific primers to detect pathogenic fungi [79].

583 Other platforms are available for the diagnosis of invasive fungal infections, in particular
584 using nucleic acid amplification methodologies, as for example the LightCycler® SeptiFast and the
585 SepsiTest™ [48,73], being these some of the most frequently used in hospital microbiology.

586 LightCycler® SeptiFast is a platform developed based on the multiplex real-time PCR
587 methodology, capable of detecting, in 6 hours, 19 bacteria species and 6 fungal species (5 *Candida*
588 spp. and *Aspergillus* fumigatus [80]), directly from clinical samples [48]. The identification of the
589 pathogens is accompanied by the software already incorporated in the equipment, the SeptiFast
590 Identification [48,81–83]. This methodology is already commercially available in Europe, even
591 though not yet in the United States of America. The disadvantage linked to its use is that it is not
592 possible to quantify the identified pathogen, which is essential to ensure the severity of the infection
593 [48]. Korber and colleagues [80] aimed to compare the effectiveness of the platform in identifying
594 fungal pathogens in clinical samples, with fungal cultures. It was reported that SeptiFast was able

595 to detect 98 of the 120 pathogens, through clinical samples, while fungal cultures were only
596 positive for 63 of the 120 pathogens. Results showed that SeptiFast was able to provide more
597 accurate detection of the pathogenic species when compared to fungal cultures, and since it is a
598 fully automated platform it can be used in clinical context [80].

599 SepsiTest™ is a platform that combines panfungal PCR with the sequencing of amplicons
600 [48]. In this way, the methodology uses universal primers that amplify the 18S region of the fungal
601 species rRNA, followed by sequencing of PCR products [48,84,85]. This methodology can be used
602 directly from clinical samples, using 1 mL of whole blood, or other sterile fluids, allowing results
603 in 8 hours. This platform was able to identify several positive samples about 13 to 75 hours before
604 blood cultures [84,85].

605 Table 2 was compiled to summarize the real-time PCR-based methodologies commercially
606 available, reviewing their most important features.

607

608 **4.3 Novel methodologies**

609 Recently, combinations of the most innovative and positive aspects of various
610 methodologies have emerged, to ensure a quick and efficient diagnosis [48,73]. Scientific
611 advances, which have been felt in recent decades, were the main driving force behind the
612 emergence of these combined methodologies, gathering several advantageous in a single
613 methodology. Some examples are the Sepsis Flow Chip platform (real-time PCR combined with
614 reverse dot blot hybridization), and ePlex® (PCR combined with electrochemical examination),
615 which were previously described in this review. However, new methodologies for the diagnosis of
616 fungal species continue to appear, some emerging from the positive aspects of previous
617 methodologies, and others with a completely innovative character.

618

619 **4.3.1. *Candida* panel and filamentous fungi panel**

620 *Candida* panel and filamentous fungi panel is a recent technique proposed by Carvalho-
621 Pereira *et al.* [86] based on a multiplex PCR methodology coupled with capillary electrophoresis,
622 for the separation of PCR products, and product size determination by GeneScan. *Candida* panel
623 uses specific primers to identify the 5 most common species related to infections by *Candida*, and
624 the Filamentous Fungi Panel uses specific primers that identify the most prevalent species in
625 infections caused by *Aspergillus* and *Rhizopus arrhizus*. The diagnosis is made through the
626 visualization of the panel, based on the appearance of peaks. Each peak corresponds to a different
627 PCR product size, which, in turn, is associated with a specific species. The innovative character of
628 the work developed is the use of specific primers that result in different and specific amplicon
629 lengths for each species combined with different fluorochromes. This allows a practical and direct
630 interpretation of the results by the visualization/identification of the specific amplicons in the panel.
631 Although not yet commercially available, the methodology showed a sensitivity of 89% and
632 specificity of 100%, when using whole blood or serum [86].
633
634 **4.3.2. Solid-phase cytometry**

635 Solid-phase cytometry emerged from the combined use of two existing methodologies,
636 fluorescence microscopy, and flow cytometry. This innovative methodology allows the detection
637 and quantification of various microorganisms, such as fungi and bacteria [87]. This methodology
638 delivers fast results, in a fully automated way, with sufficient sensitivity and specificity to diagnose
639 an infection, directly through clinical samples. However, solid-phase cytometry still faces some
640 obstacles in clinical microbiology laboratories, especially associated with the validation and
641 verification of the methodology [87]. Therefore, it is commonly used in food, water, and air quality
642 control trials [87,88].

643 Until the final result of the microorganism identification, the sample goes through a series
644 of steps [89]. The sample is first filtered on a membrane and then retained cells are fluorescently
645 labelled. Fluorescent cells are analyzed using a solid-phase cytometer, where background signals
646 are distinguished from specific signals referring to target cells. Finally, the sample is analyzed
647 using fluorescence microscopy, in order to validate and examine the target cells [87,89].

648 In a study conducted by Lies *et al*. [88], solid-phase cytometry methodology was used to
649 identify *A. fumigatus* in air samples, since the control of spores in the air is an important
650 epidemiological factor. The results obtained through this methodology presented several
651 advantages when compared to traditional culture methods. Solid-phase cytometry has a low
652 detection limit (4 cells per m^3), results within 24 hours, and high sensitivity and specificity for *A.*
653 *fumigatus* [88].

654 The effectiveness of solid-phase cytometry was also analyzed in clinical samples, with the
655 objective of identifying *Candida* cells present in the whole blood of patients diagnosed with a
656 possible systemic infection [89]. Despite the low number of clinical samples used in the study,
657 several advantages of this methodology when compared to blood cultures are described. Solid-
658 phase cytometry was able to provide faster results, and also an accurate quantification of *Candida*
659 cells. This methodology was also able to identify mixed infections, present in 5 of the 16 clinical
660 samples used, which suggests that it is a more common phenomenon than the one that diagnosis
661 through blood cultures suggests [89].

662

663 **4.3.3. Fourier Transform InfraRed (FTIR)**

664 The Fourier transform infrared (FTIR) methodology, is the most used technique in
665 microbiology laboratories, having in its basis the principles of spectroscopy. This methodology has
666 several applications, from soil and water quality control trials, to industrial applications in

polymers, and also clinical applications in biological samples [90]. The functionality of this methodology is based on passing infrared radiation through the sample, where some radiation ends up being absorbed. The equipment's detector produces a spectrum that represents the molecular fingerprint of the analyzed sample. In clinical terms, different microorganisms will produce different fingerprints, and their distinction is possible through the analysis of the spectra produced [90].

Potocki and co-workers [91] used FTIR methodology with the main objective of distinguishing *Candida* non-*albicans* from *C. albicans* species, since non-*albicans* species are mostly associated with resistance to antifungal agents used. FTIR was used in 25 clinical isolates of *Candida* spp. and the identification and distinction of each isolate were possible due to the diversity of spectra produced by each species. The methodology also appears promising regarding the search for antifungal resistance genes, since resistant species will produce a different spectrum than a non-resistant species [91]. According to Erukhimovitch [92], the distinction between a bacterial and fungal infection remains a problem, especially due to the symptomatic similarities. Generic antibiotics are often administrated before the results of blood cultures are analyzed, taking about 2 to 5 days to grow, and in some fungal pathogens up to two weeks. Therefore, FTIR methodology is considered a great screening tool in these situations since bacteria and fungi produce completely different spectra [92]. In the study, clinical samples were used to distinguish bacterial from fungal infections. The results show that this distinction was possible in just 1 hour, which turns out to be a huge advantage over blood cultures [92].

4.3.4. Surface-Enhanced Raman Scattering (SERS)

Surface-enhanced raman scattering (SERS) is a combination of Raman spectroscopy and the use of nanoparticles, which has been previously used to detect several pathogenic organisms,

including fungi. This technique provides qualitative and quantitative analysis, allows to trace clinically relevant biomolecules, and establishes molecular profiles that can be important to determine the severity of fungal infections [93]. Moreover, a recent study conducted by Hu *et al.* [94] aimed to directly detect and identify *Candida* species in serum, by combining nanoparticles, SERS spectrum, and OPLS-DA multivariate statistical analysis. In this experiment, Fe3O4@PEI magnetic nanoparticles showed high capture efficiency of *Candida* cells in serum, due to electrostatic attraction, producing the Fe3O4@PEI*Candida* complex. Then, positively charged silver nanoparticles (AgNPs+) were used as the substrate for SERS, to enhance the intensity of the signal. This method is described as fast, affordable, and non-destructive, as does not require pure cultures, cell wall lysis, or DNA extraction [94].

4.3.5. Nanotechnology

Nanotechnology has increasingly contributed to the development and evolution of health-related fields. For instance, the application of gold nanoparticles has been intensively studied, being applied in vaccines as preventive agents, used as drug delivery systems in cancer or other health conditions therapies, and also in diagnostic approaches [95]. Sojinrin and co-workers [96] developed a protocol to detect the presence of spore-forming fungi based on gold nanoparticles. Essentially, when the gold nanoparticles enter in contact, for example, with *Aspergillus niger*, they endure structural and morphological changes, from spherical to star-shaped, and change of color from red to blue. This is a fast, straightforward and low-priced method, yet does not allow specific identification of pathogens [96].

4.3.6. Nuclear Magnetic Resonance (NMR)

714 Since 2001, NMR has been useful in the microbiology field for species identification and
715 detection, through the use of nanoparticles, with subsequent analysis by magnetic resonance [48].
716 In this case, the detection of the target organism is done by beads that have a complementary
717 sequence to the organism's DNA, allowing the binding. This binding allows the aggregation of
718 beads, which can be observed through magnetic resonance. NMR methodologies can be used alone,
719 or following a conventional PCR, for product analysis [26,27]. T2Candida® was the first
720 methodology to be verified and validated by the FDA (Food and Drug Administration) for invasive
721 candidiasis diagnosis. It is an automated platform based on NMR, which allows to detect and
722 identify 5 *Candida* spp. directly from clinical samples of whole blood or serum, within 3 to 5 hours
723 [48,73]. Firstly, the clinical sample is inserted into the platform, yielding an automated DNA
724 extraction, which is then analyzed by magnetic resonance, detecting pathogenic *Candida* spp. [97–
725 99]. In the clinical trial study, T2Candida® demonstrated a sensitivity of 91.1% and specificity of
726 99.4% which was a major achievement regarding molecular diagnosis [99].
727

728 **4.3.7. Biosensors**

729 Other research area under constant development consists in the use of biosensors. Those
730 are designed as portable devices that convert biological and biochemical information into an output
731 analytical signal [100]. Fungal biosensors produced for clinic diagnosis have to fulfil several
732 requirements, such as the careful selection of a specific biomarker of the target pathogen, which
733 has to be suitable for the biological recognition system and to hold measurable features associated
734 with normal conditions or with infection [100]. Pla *et al.* [101] described an innovative nanosensor
735 to detect *C. auris* based on biocompatible nanoporous anodic alumina (NAA) supports, with the
736 pores loaded with fluorophores and oligonucleotides attached. The oligonucleotides are specially
737 selected in order to make the sensor completely specific for *C. auris*. When this pathogen is present

738 in a sample, the oligonucleotide hybridizes to its genomic DNA exclusively, thus opening the pore
739 and releasing the trapped fluorophore. This system presents high sensitivity and selectivity, the
740 results can be obtained within an hour, and previous steps such as DNA extraction are not required
741 [101].
742

743 **4.3.8. Volatile Organic Compounds (VOC) assay**

744 Volatile organic compounds assay is a new type of methodology for the diagnosis of
745 invasive aspergillosis, with sensitivity rates above 90%. In this assay, several metabolites
746 characteristic of *A. fumigatus* are detected from the patient's exhaled air [27,102]. The innovative
747 character of this assay is that it uses an artificial olfactory system that distinguishes several VOCs
748 produced by the pathogen, called "breathprints" [102–104]. The majority of VOCs produced by *A.*
749 *fumigatus* that are identified by this assay are 3-octanone, 2-pentylfuran, isoamyl alcohol, ethanol
750 and others [105,106]. However, the detection of these metabolites is often associated with
751 pulmonary diseases, in this case, pulmonary aspergillosis [104].
752

753 **5. Conclusions and final remarks**

754 The scientific community has played a very important role in improving diagnostic
755 methodologies in order to achieve accurate detection and identification of clinically relevant fungal
756 pathogens. This development was mainly due to technological advancements in the last two
757 decades, but also to the greater knowledge of molecular genetics. Another fundamental factor is
758 the increasing interaction between humans and wildlife, which enhances the appearance of new
759 pathogenic species.

760 Real-time PCR methodologies are becoming increasingly more valued for the diagnosis of
761 fungal infections. This preference is mainly due to the easy handling of the methodology, and also

762 because the reaction occurs in a closed system, which makes external contamination more difficult.
763 For those reasons, the real-time PCR methodology remains the most widely used in the hospital
764 environment for diagnosing numerous infectious diseases.

765 Regarding the identification of fungal pathogens, it is of utmost importance to achieve
766 specific identifications, in order to establish an adequate therapeutic plan, increasing the patient's
767 chance of survival. In the treatment of systemic fungal infections, identification at the species level
768 is essential, because different fungal species have distinct antifungal susceptibilities. Therefore, a
769 specific antifungal, with a specific concentration should be used. For example, *C. auris* is resistant
770 to the majority of antifungals, *C. glabrata* easily acquire resistance to fluconazole, and *C. krusei*
771 has intrinsic resistance to azoles.

772 The development of more sophisticated and automated molecular methodologies that
773 delivers faster results represents a huge improvement in the clinical management of fungal
774 infections. However, there is a long way to go to accomplish the global standardization of such
775 methodologies.

776

777 **Declaration of competing interest**

778 The authors declare no conflict of interest.

779

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786

787 **Table 1.** Overview of advantages and disadvantages of the most commonly used methodologies
788 for fungal infections diagnosis.

	Methodologies	Advantages	Disadvantages	References
***Proven* diagnosis**	**Fungal culture**	- Detection of the fungal pathogen; - Detection of antifungal resistance; - Identification at species level.	- Long turn-around-time; in case of yeasts, up to five days, and molds up to two weeks; - Long-delayed targeted treatment; - Prone to contaminations; - Low sensitivity for candidemia and aspergillosis.	[27,107]
	Microscopy	- Visualization of fungal structures; - Analysis of shape, tracking of motion, and classification of microorganisms; - Visualization of fungal biofilm formation.	- Does not allow fungal genus or species identification; - Similar microscopic appearance of several fungus.	[26,27,108]
	Histopathology	- Detection of tissues invasion by fungi; - Detection of the host response or tissue necrosis.	- Similar histopathologic appearance of several fungus; - The use of stains does not always provide an accurate identification at species level; - Limited sensitivity.	[26,27]
	Chromogenic media	- Detection in polymicrobial samples; - Several commercially available chromogenic media; - Detection and identification of *Candida* at the species level; - Fast and cost-efficient.	- Difficult distinction between *Candida* non-*albicans* species.	[40]

	Fluorescence *in situ* hybridization (FISH)	- Accurate identification of *Candida* spp. infections; - Time saving, comparing with conventional methods; - Applied to measure the gene expression; - High specificity and sensitivity.	- Low detection limit; - Reduced number of peptide nucleic acid (PNA) probes commercially available.	[26,109]
	Mass spectrometry-based methods	- Identification of the pathogen at the genus, species, and strain levels; - Accurate and rapid identification of *Candida* spp. and *Aspergillus* spp.; - High concordance with conventional methods; - Easy performance; - Reduced cost per analysis; - Applicability for a wide range of microorganisms.	- Prior extraction step is required; - Incapable of performing quantification; - High initial instrument cost.	[26,27,110]
***Probable* diagnosis (Serological methods)**	**1,3 β-D-glucan**	- Detection of relevant fungal pathogens; - Non-invasive; - Fast results; - Repetition of serum samples analysis led to increased specificity.	- Nonspecific panfungal test; - Lower sensitivity in patients with hematologic malignancies and bacterial infection; - Certain fungus produce less β-D-glucan (*Cryptococcus* spp.) or do not produce any (*Blastomyces* spp. and *mucoraceous* moulds); - Lack of specificity for endemic mycosis diagnosis.	[27,100,111]
	Mannan antigen and antimannan antibody	- Good specificity and sensitivity when combined; - Non-invasive; - Economic; - Deliver fast results.	- Decreased specificity and sensitivity due to previous antibiotic and antifungal treatments, respectively;	[27,100,111]

			- Low sensitivity for *Candida krusei* and *Candida parapsilosis.*	
	Galactomannan	- Good biomarker for the detection of invasive aspergillosis; - Useful for assessing the response to antifungal therapy.	- Low sensitivity for early diagnosis.	[111]
	Antibody-based (Immunofluorescence, ELISA, Lateral flow assay, Latex agglutination assay)	- Higher accuracy than the standard serologic markers mentioned above; - Serologic markers; - Low cost; - Easy and fast performance.	- Reduced sensitivity for immunocompromised patients; - Limited specificity; - Antigen-antibody methods still not available for some fungal pathogens (mucormycosis, fusariosis, and scedosporiosis).	[27,108]
***Probable* diagnosis (Molecular methods)**	**Nuclear magnetic resonance (NMR)**	- Quantitative method; - Reduced sample-result time; - Promising combination of NMR with PCR to direct detection and identification of *Candida* spp. from blood samples (T2 *Candida*).	- Reduced sensitivity and low limit of detection.	[26,27,97,99]
	PCR-based methods	- Short turnaround time; - High sensitivity and specificity; - Real-time PCR allows quantification of amplified DNA in real-time; - Allows species identification and intraspecies differentiation.	- Traditional PCR does not allow quantification of the amplified DNA; - Lack of standardization of the fungal DNA isolation techniques; - Contaminations; - Careful selection of primers and optimization of the reaction conditions.	[24,26,27,73]

789

790 **Table 2.** List of commercially available real-time PCR-based assays for detection of fungi.

Product (Manufacturer)	Assay method	PCR targets	Detected species	Detected resistance mutations	Specimens	Assay Time	Sensitivity / Specificity[a]	References
SeptiFast LightCycler (Roche)	Multiplex Real-time PCR (DNA melt curve analysis)	ITS region	- *Candida albicans* - *Candida tropicalis* - *Candida parapsilosis* - *Candida krusei* - *Candida glabrata* - *Aspergillus fumigatus*	-	WB	6-7 h	60 – 86% / 96.1– 100%	[112–115]
Magicplex Sepsis Real-Time Test (Seegne)	Multiplex real-time PCR	Unknown	- *Aspergillus fumigatus* - *Candida albicans* - *Candida glabrata* - *Candida krusei* - *Candida parapsilosis* - *Candida tropicalis*	-	WB	6 h (including DNA extraction)	29% / 95%	[116,117]
***A. fumigatus* Bio-Evolution (Bio-Evolution)**	Real-time PCR	ITS1 region	- *Aspergillus fumigatus*	-	BAL	<80 minutes (excluding DNA extraction)	81% / 100%	[118,119]
MycAssay *Aspergillus* (Myconostica)	Real-time PCR with molecular beacons	18S rDNA	Eighteen *Aspergillus* species including: - *Aspergillus fumigatus* - *Aspergillus flavus* - *Aspergillus terreus*	-	Serum BAL	4 h (after sample collection)	80 – 100% / 82.4 – 98.6%	[112,119–121]

			- *Aspergillus niger*					
AsperGenius® (PathoNostics)	Multiplex real-time PCR	28S rRNA	*Aspergillus* spp. including: - *Aspergillus fumigatus* - *Aspergillus terreus*	Cyp51A gene: - TR34 /L98H amino acid substitution - TR46 /Y121F /T289A amino acid substitutions	BAL Serum Plasma Biopsy CSF	<3h (after sample collection)	65.5 – 88.9% / 77.8 – 93.3%	[112,119,122–125]
Fungiplex® *Aspergillus* and Fungiplex® *Aspergillus* Azole-R (Bruker Daltonics)	Multiplex real-time PCR	Unknown	- *Aspergillus fumigatus* - *Aspergillus flavus* - *Aspergillus niger* - *Aspergillus terreus*	Cyp51 gene: - TR34 / L98H amino acid substitution - TR46 / T289A and Y121F amino acid substitutions	WB Serum Plasma BAL	2 h (excluding DNA extraction)	60% / 91.2%	[126,127]
***Aspergillus* spp. ELITe MGB® Kit (ELITechGroup)**	Quantitative real-time PCR	18S rDNA	*Aspergillus* spp. including: - *Aspergillus niger* - *Aspergillus nidulans* - *Aspergilus terreus* - *Aspergillus flavus* - *Aspergillus versicolor*	-	BAL BA	NA	90 – 100% / 97 – 97.8%	[128,129]

			- *Aspergillus glaucus*					
MycoReal *Aspergillus* (Ingenetix)	Real-time PCR (melt curve Analysis)	ITS2 region	-*Aspergillus fumigatus* -*Aspergillus flavus* - *Aspergillus nidulans* - *Aspergillus niger* - *Aspergillus terreus*	-	BAL Blood CSF Tissues	NA	NA	[121,130,131]
MycoGENIE® *Aspergillus* Species and MycoGENIE® *Aspergillus fumigatus* and resistance TR34/L98H (Ademtech)	Quadruplex real-time PCR	28S rRNA	*Aspergillus* spp. including: - *Aspergillus fumigatus*	TR34/L98H mutations	Serum BAL Biopsy	NA	71 – 100% / 84.6 – 100%	[127,132,133]
***Asp*ID (OlmDiagnostics)**	Multiplex real-time PCR	Unknown	*Aspergillus* spp. including: - *Aspergillus terreus*	-	BAL	90 minutes (excluding DNA extraction)	94.1% / 76.5%	[132–134]
***Cand*ID® and *Auris*ID® (OlmDiagnostics)**	Multiplex real-time PCR	Unknown	*Cand*ID: - *Candida albicans* - *Candida dubliniensis* - *Candida glabrata* - *Candida krusei* - *Candida parapsilosis* - *Candida tropicalis* *Auris*ID: - *Candida auris*	-	*Cand*ID: Plasma Synthetic BAL *Auris*ID: Blood	45 min (excluding DNA extraction)	*CandID:* NA *Auris*ID: 96.6% / 100%	[117,135]

FungiPlex® *Candida* (Bruker Daltonics)	Multiplex real-time PCR	Unknown	- *Candida albicans* - *Candida parapsilosis* - *Candida dubliniensis* - *Candida tropicalis* - *Candida glabrata* - *Candida krusei*	-	WB Serum Plasma	<2 h (excluding DNA extraction)	98.4 – 100%/ 94.1 – 99.8%	[115,117]
PneumoGenius (PathoNostics)	Real-time PCR	Mitochondrial ribosomal large subunit (rLSU) and two dihydropteroate synthase (DHPS) gene mutations	-*Pneumocystis jirovecii*	DHPS mutations: - codon 55 - codon 57 Point mutations: - 165 (Thr55Ala) - 171 (Pro57Ser)	BAL	<3 h (after sample collection)	70% / 82%	[134,135]
AmpliSens *Pneumocystis jirovecii* (carinii)-FRT (AmpliSens)	Real-time PCR	Mitochondrial large subunit ribosomal(rLSU) RNA gene	-*Pneumocystis jirovecii*	-	BAL BA Biopsy	130 min (excluding DNA extraction)	100% / 83%	[136]
***Pneumocysist jiorovecii* Bio-Evolution (Bio-Evolution)**	Real-time PCR	Unknown	-*Pneumocystis jirovecii*	-	BAL BA	80 min (excluding DNA extraction)	72 - 95% / 82 - 100%	[136,137]
PneumID® (OlmDiagnostics)	Multiplex real-time PCR	Unknown	-*Pneumocystis jirovecii*	-	BAL BA	45 min	-/ 90%	[138]

						(excluding DNA extraction)		
MucorGenius® (PathoNostics)	Real-time PCR	Unknown	-*Rhizopus* spp. -*Mucor* spp. -*Lichtheimia* spp. -*Cunninghamella* spp. -*Rhizomucor* spp.	-	BAL Biopsy Serum	<3 h (after sample collection)	75 – 90% / 97.9%	[139–141]

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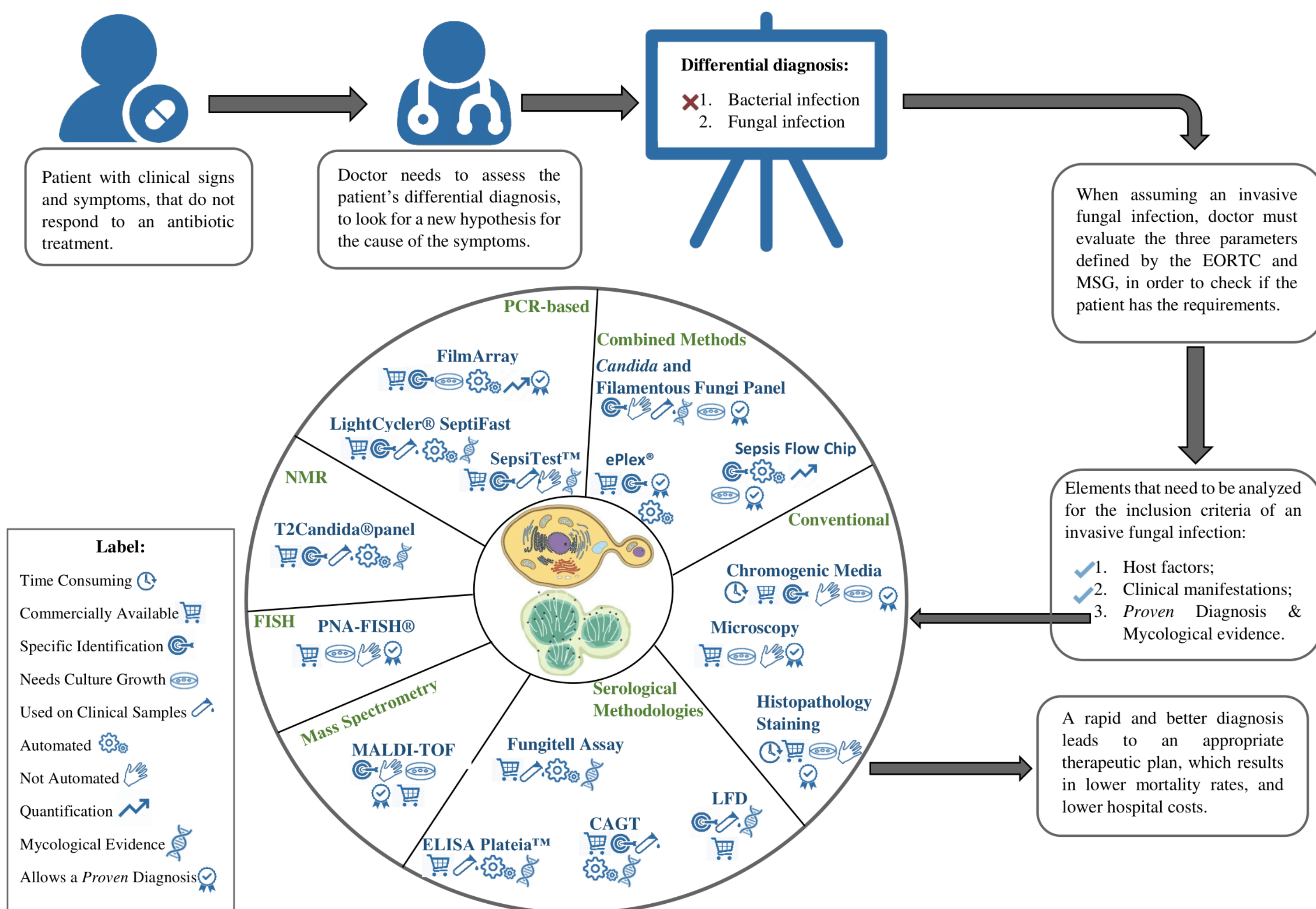
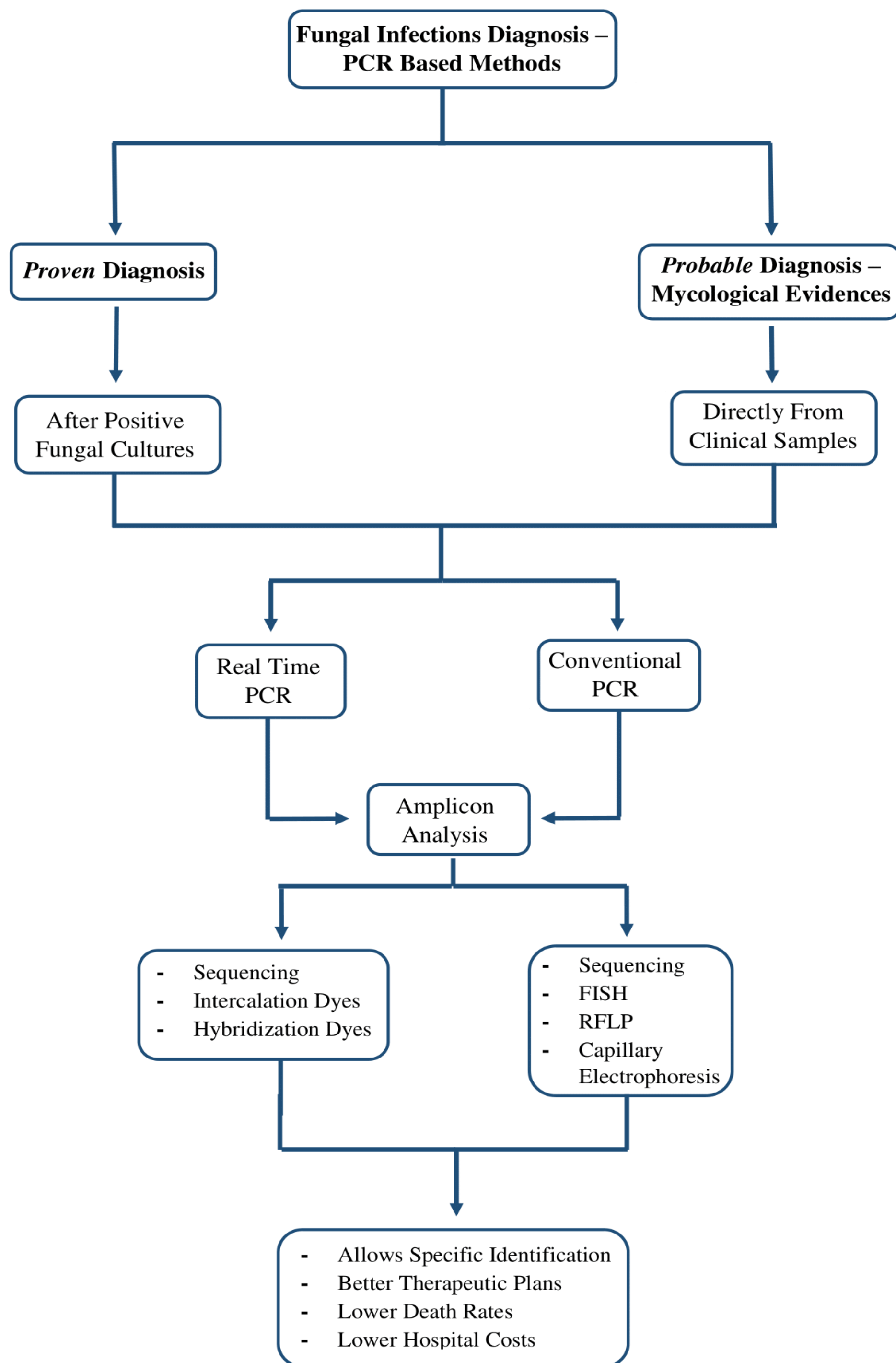
[a]Sensitivity and specificity vary according to the specimen, as well as the clinical context of the patients.
Abbreviations: BA, bronchial aspirate; BAL, bronchoalveolar lavage; CSF, cerebrospinal fluid; WB, whole blood; NA, not available.



Abbreviations: EORTC, European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group; MSG, Mycoses Study Group; NMR, Nuclear Magnetic Resonance; FISH, Fluorescence *in situ* hybridization; CAGT, *Candida albicans* Germ Tube Antibody Assay; LFD, Lateral-Flow Devices; MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization – Time of Flight.

Figure 1. Systemic fungal infection diagnosis workflow. When a patient does not respond to the antibiotic treatment, a systemic fungal infection should be included in the differential diagnosis. After evaluating the 3 parameters defined by the EORTC and MSG (host factors, clinical manifestations and mycological evidence), and if there is a strong evidence for a systemic fungal infection, tests are carried out. There are several methods to achieve a *proven* diagnosis, however these methods delay the patient's treatment, which can also lead to more hospital costs. On the other hand, other methodologies provide a *probable* diagnosis, which means that only traces of the pathogen are detected, nevertheless these methodologies are capable of providing an accurate and faster result, which leads to a better therapeutical plan and lower hospital costs.



Abbreviations: PCR, Polymerase Chain Reaction; FISH, Fluorescence *in situ* Hybridization; RFLP, restriction fragment length polymorphism

Figure 2. PCR-based methods workflow for fungal infections diagnosis and the possible outcomes.

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Figure 1

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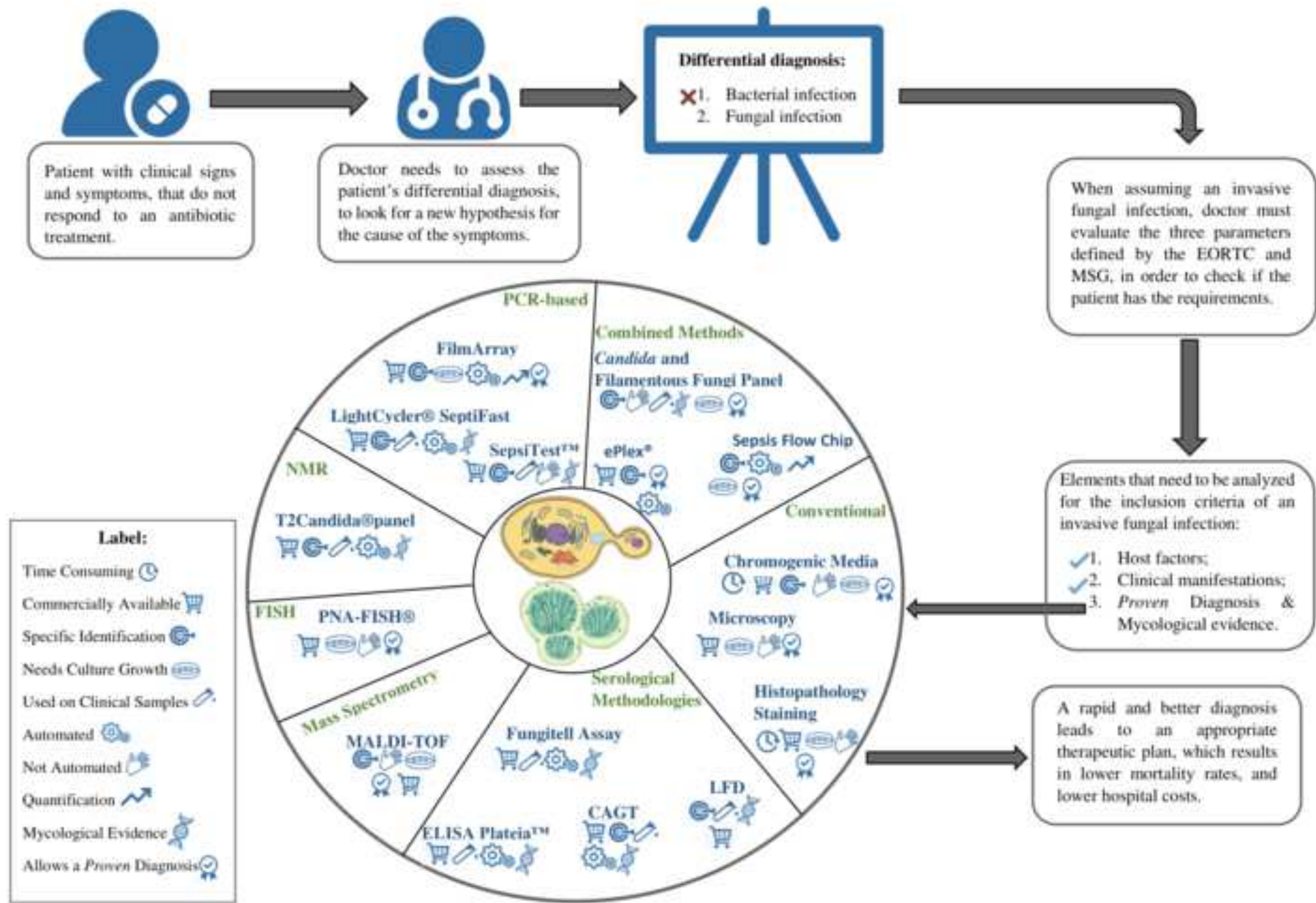

Figure 2

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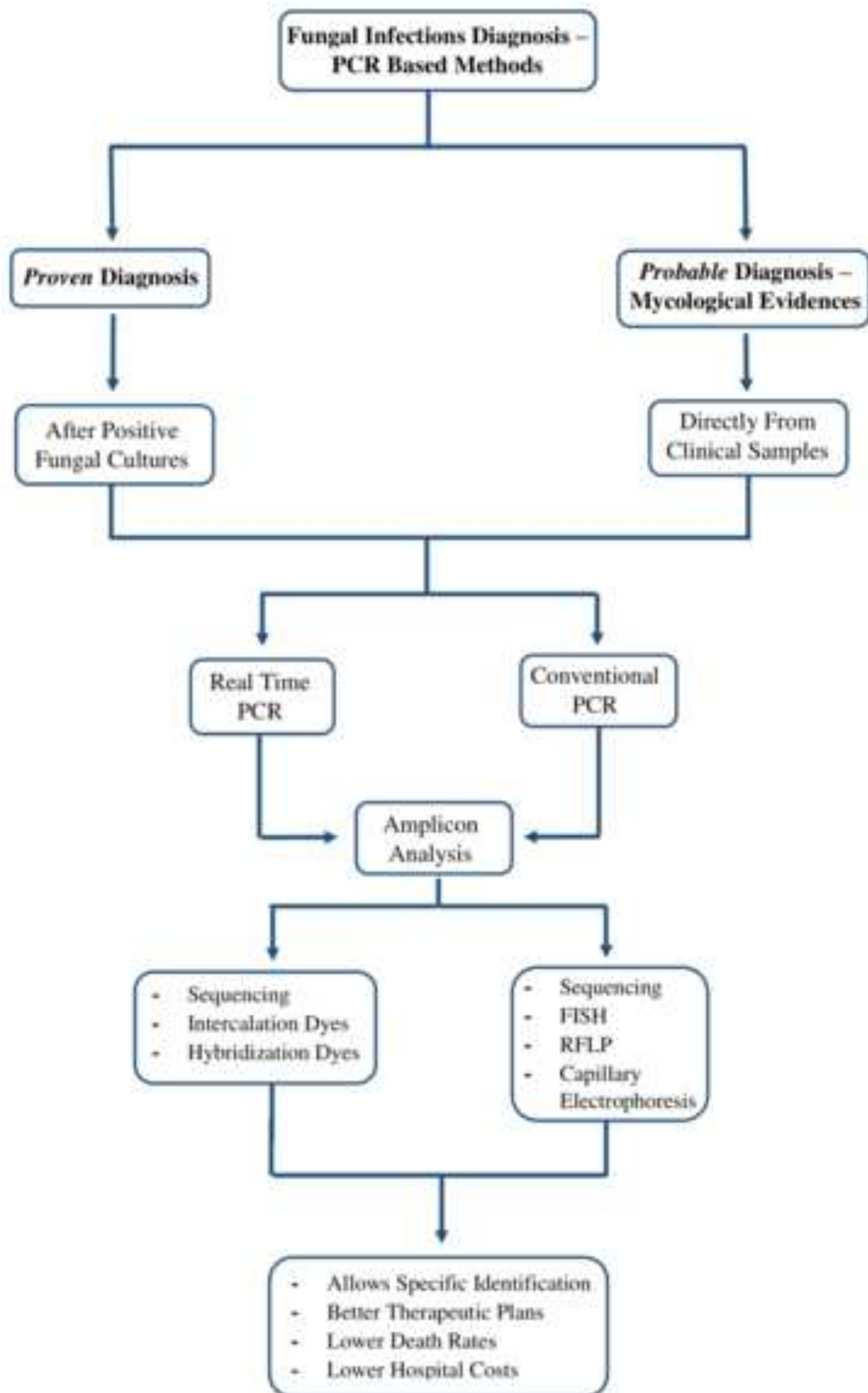

Cover Letter

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Universidade do Minho
Escola de Ciências
Departamento de Biologia

Research in Microbiology Editorial Office

August 16th, 2021

Subject
Manuscript submission

Message

Dear Editor of Research in Microbiology,

I herewith would like to submit our original review manuscript entitled "Fungal Infections Diagnosis – Past, Present and Future", by Alexandre Mendonça, Helena Santos, Ricardo Franco-Duarte and Paula Sampaio.

The objective of the work herein submitted was to review the available methods to diagnose fungal infections, comparing them and evaluate their potential. We believe that despite the tremendous advances obtained in the last years in fungal infections diagnostic methods, they still lack standardization before becoming routinely used in hospital laboratories. This can be perceived now, as PCR-based methodologies have proved to be an essential tool fighting against the COVID-19 pandemic. In our review, we assessed all the main steps of the diagnosis of a systemic fungal infection, and compared the standardized methods with the more "futuristic" ones. All the collected information allowed us to compile a systemic fungal infection diagnosis workflow, which we believe will be of foremost importance for everyone trying to identify a systemic fungal infection, providing in this way more accurate and fast results, and leading to better therapeutical plans and lower hospital costs.

Being fungal infections diagnosis a very trending topic lately, we believe this review will be very well accepted and cited by researchers worldwide.

The content and authorship of the present manuscript has been approved by all authors, also as its submission to *Research in Microbiology*. The work herein submitted represents original work of all the authors, and has not been submitted earlier to this journal or any other journal.

The submission of a review article to *Research in Microbiology* was previously accepted by doctor Tarek Msadek.

I thank you for your attention, and would appreciate very much the publication of this manuscript in the journal *Research in Microbiology*.

With kind regards,

Ricardo Duarte

(Ricardo Franco Duarte)