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Review

Laboratorial diagnosis of paracoccidioidomycosis and new insights for the future of fungal diagnosis

F.R.R. Teles [a,b,*], M.L. Martins [b,c]

[a] Centre for Malaria and Tropical Diseases (CMDT), Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL), Rua da Junqueira, 100, 1349-008 Lisboa, Portugal

[b] Laboratory of Mycology/Unit of Medical Microbiology, Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL), Rua da Junqueira, 100, 1349-008 Lisboa, Portugal

[c] Centro de Recursos Microbiológicos (CREM), Universidade Nova de Lisboa (UNL), Quinta da Torre, 2829-516 Caparica, Portugal

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ABSTRACT

Paracoccidioidomycosis (PCM) is the most prevalent mycosis in Latin-America. As for other mycosis, its importance of has been largely underestimated, partially due to the limited geographical distribution of the etiologic fungal agent (*Paracoccidioides brasiliensis*). However, the advent of AIDS and other immune suppressing conditions is creating an emergent need for improved diagnostic tests envisaging simpler, cheaper, faster and more sensitive and accurate detection of pathogenic fungi, especially those causing systemic and opportunistic diseases. Routine laboratorial diagnosis of PCM disease relies mainly on direct observation of the fungus. However, culture growing is slow and, too often, definite diagnosis can only be obtained at later growing stages. Immunodiagnosis is also widely employed, although usually cumbersome and complex. Enzyme-based immunoassays are more amenable to automation for high-throughput testing, but may lead to cross-reactivity with other fungi. Plus, molecular diagnosis relying on polymerase-chain reaction (PCR) and nucleic-acid hybridization, although still at early stages of application to routine diagnosis of *P. brasiliensis*, has triggered the development of techniques for its improved specific detection, thus contributing for epidemiological studies as well. In the future, microarrays and newer biosensing technologies, coupled to new bionanotechnological tools, will certainly improve diagnosis of PCM and other mycosis through very specific and sensitive pathogen biomolecular detection.

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Contents

Abbreviations: cDNA, complementary DNA; CF, complement fixation; CNS, central nervous system; CNTs, carbon nanotubes; ELISA, enzyme-linked immunosorbent assay; FET, field-effect transistor; gp43, 43 kDa glycoprotein; gp70, 70 kDa glycoprotein; hsp60, 60 kDa heat-shock protein; hsp87, 87 kDa heat-shock protein; ID, immunodiffusion; IgA, immunoglobulin A; IgE, immunoglobulin E; IgG, immunoglobulin G; IgM, immunoglobulin M; inh-ELISA, inhibition ELISA; ITS, internal transcribed spacer; ITS1, internal transcribed spacer 1; ITS2, internal transcribed spacer 2; MIPs, molecularly imprinted polymers; NPCM, neuroparacoccidioidomycosis; p27, 27 kDa protein; PCM, paracoccidioidomycosis; PCR, polymerase chain-reaction; QCM, quartz crystal microbalance; RAPD, random amplified polymorphic DNA; rDNA, ribosomal DNA; RNA, ribonucleic acid; rRNA, ribosomal RNA; RT-PCR, reverse-transcription PCR; SAM, self-assembled monolayer; SNPs, single-nucleotide polymorphisms; SWCNTs, single-walled carbon nanotubes; UKPCG, United Kingdom–Ireland Fungal PCR Consensus Group.

* Corresponding author at: Centre for Malaria and Tropical Diseases (CMDT), Instituto de Higiene e Medicina Tropical (IHMT), Universidade Nova de Lisboa (UNL), Rua da Junqueira, 100, 1349-008 Lisboa, Portugal. Tel.: +351 213652600x318/305; fax: +351 213622458/213632105.
E-mail address: fteles@ihmt.unl.pt (F.R.R. Teles).

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1. Introduction

The recognition of mycosis as potential causative agents of severe infectious diseases has been highly underestimated by clinicians, but the advent of HIV/AIDS and of other immunodepressing conditions that modern life increasingly imposes to mankind have drastically changed this situation, given the huge increase of immunocompromised patients among the human population and thus their susceptibility for opportunistic infections. PCM, also known as South-American blastomycosis, is caused by the fungus *Paracoccidioides brasiliensis* and is one of the most prevalent systemic mycosis in Latin-America, especially in Brazil. Even so, the real incidence of PCM in Brazil is still underestimated because case notification is not obligatory [1]. Although uncommon, some cases have also been recently reported in Europe, both in individuals from endemic regions and in returning travelers from those areas [2,3]. While millions of individuals may be infected worldwide, only a small percentage actually develops disease [4]. PCM ranks among the most deadly infectious diseases and has the highest mortality rate among all systemic mycosis. The dimorphic, temperature-dependent transition of *P. brasiliensis* from the mycelial to the yeast form is critical for pathogenicity [5], with primary sources of infection being probably air-borne fungal particles. There are no reports about horizontal transmission between humans, but living reservoirs include several domestic and wild animals (*e.g.* dogs and armadillos) [6,7]. The lack of experienced clinicians and the long latent period of the disease may significantly contribute to the difficulty of diagnosing PCM outside endemic regions. Allied to the effects of global climate warming, to the rapid and significant sociodemographic and economical changes currently taking place in Brazil and to the intense immigration flows from South-America to Europe and USA, this may ultimately lead to the emergence of PCM as a problem of public health in the receptor regions. In addition, PCM patients frequently exhibit other diseases, including tuberculosis, carcinomas, enteroparasitosis, pulmonary infections, AIDS, leishmaniosis, leprosy, Chagas disease and other mycosis. In general, these and other co-morbidities must be considered for differential diagnosis of PCM, as rapid and accurate diagnosis is essential to timely begin specific therapy. Conventional diagnosis of fungal infections usually begins with the identification of clinical symptoms followed by laboratorial direct examination by microscopy and isolation from clinical specimens, which is still the "gold-standard" for laboratorial diagnosis of fungal infections. Despite the usual high sensitivity of this method, atypical forms of *P. brasiliensis* may be mistaken with those of other fungi [8]. This approach also requires skilled manpower, long times for culture growing and ability of the fungus to be cultivated *in vitro*, besides the drawbacks of being non-quantitative and biohazard. Plus, a positive culture may indicate fungal colonization instead of true invasion, especially in the case of opportunistic microorganisms. Serological techniques are usually simpler than culture and very useful for diagnosis and follow-up of infected patients, but may suffer from cross-reactivity between the anti-*P. brasiliensis* antibodies and other fungi antigens [9]. The sensitivity of serology may still be low in immunocompromised patients, where only low levels of circulating antibodies exist. In general, serology is also inadequate to distinguish between different microorganism strains, especially from different geographic regions [10]. Molecular diagnosis has brought a new and improved range of essentially quantitative techniques. Molecular methods, usually relying on prior genomic amplification by PCR and other related techniques, are essential to distinguish and identify circulating genotypes, and have shown particular success in detecting *P. brasiliensis* when microscopic observation fails and when antigen or antibody blood levels are too low for successful immunodiagnosis. A strong handicap, however, is the absence of more systematic evaluation and standardization of PCR-based tests for fungal disorders. Furthermore, positive-tested samples do not always correspond to current infections (it may be a latent infection or simple colonization) and, among these, not all lead to disease. By their ability to assess and quantify gene expression patterns, microarrays can overwhelm this limitation. These high-throughput bioanalytical systems can provide multiplexed qualitative and quantitative testing of very small volumes of complex environmental samples containing hundreds or thousands of different species. However, complexity, expensiveness and requirement for skilled technicians are usual limitations of microarrays towards widely implementation for routine laboratorial analysis. Thus, an urgent need remains to develop new and improved diagnostic tests for sensitive, specific, reproducible, simple and cheap *in situ* detection of endemic and opportunistic pathogenic fungi. In particular, the lack of sensitivity of current diagnostic methods for clinical detection of medically important fungi has certainly been responsible for the underestimated burden of many mycosis [11]. In this scope, biosensor technology is expected to provide suitable methodologies for fungal detection and diagnosis of mycosis, by suitable integration of the biorecognition event with the physical transducer and permanent fixing of the biological components into the assay device. In parallel, the emergent field of nanotechnology has rendered a multitude of new analytical procedures, structures and systems for high-performance biosensing, namely in terms of improved sensitivity and selectivity. Unfortunately, this area is still in the early stages of development and application for fungal diagnosis. This paper reviews the state-of-the art of laboratorial diagnosis of *P. brasiliensis*, encompassing direct detection, immunodiagnosis and molecular methods. It also describes noteworthy remarkable advancements on fungal diagnosis based on proof-of-concept biosensors, while pointing out new trends in bioanalysis and biomolecular diagnosis.

2. The importance of specific laboratorial diagnosis

PCM may be fatal if untreated. Like in most of the other invasive mycosis, the fungus is difficult to eliminate from the human body, and prompt detection and treatment are usually decisive to avoid long-term deleterious injuries and consequences. Unlike residents in endemic areas, immigrants develop severe disease more frequently, probably due to a lack of immunity acquired from previous exposure [12]. In addition, white persons, followed by yellow ones, seem to be more prone to the disease than natives from endemic

areas. The occurrence of PCM cases in individuals that have never left cities within endemic areas shows a progressive dissemination of this infection from rural to urban environments [13]. Outside the endemic regions, most of the reported cases worldwide refer to individuals that lived or visited Latin-America at least once, thus justifying the consideration of PCM as a travelers' disease [14]. Nevertheless, it has been reported the increasing frequency of notified cases even outside endemic areas, particularly in Europe [15]. Ironically, it has also been speculated that the increasing immigration flow towards the cities should cause a decrease in the overall disease prevalence, probably assuming its rural origin. Apart the rural origin of many inhabitants of Latin-American cities, many of these urban centers are still relatively new; therefore, it is difficult to find people in Latin-America, even in the cities, who have never directly contacted with soil and vegetation, the natural habitats of *P. brasiliensis*. As a consequence, it may thus be almost irrelevant the rural or urban provenience of Latin-America immigrants to Europe and other hosting regions in the world. Inversely, but in the same line of thought, there are documented cases of European immigrants that, upon arriving and establishing in Latin-American cities (without apparent contact with rural environments) within PCM endemic regions, became infected with PCM [16], a serious sign about the risk for the expansion of this disease to the cities, not only within the current endemic regions and countries. In particular, the high flows, during the last decades, of Japanese immigrants in Brazil – most of them rural workers – back to their country [17] should concern Japanese authorities about the possible sudden of PCM cases in this Asian country along the next years. Although certain clinical features are regularly observed in PCM patients, there is no fixed pattern or characteristics. Clinical examination may thus reveal a patient in a good condition or almost cachectic. In addition, several months usually elapse between the onset of symptoms and medical consultation. The disease may arise in the acute/sub-acute and chronic clinical forms. In the acute/sub-acute form, both genders are described to be equally affected. There is a rapid evolution affecting mainly the organs of the reticuloendothelial system, with multiple adenopathies and sharped collapse of the general health status (with fever, anorexia and anemia). The fungus can disseminate to skin and other tissues. Although the lung compromise is usually asymptomatic, the fungus can be isolated from sputum. As in immunocompromised patients, usually the pulmonary system is minimally affected. Late reactivation phenomena, probably associated with immunedepression factors, may result in chronic infection [11], which is more common and less severe than the acute form [18] and accounts for more than 90% of the infected patients [19]. The chronic form is especially incident within middle-age males, taking months or years to become established. Several organs may be compromised (with uni- or multifocal origin), including compromise of the oral mucosa [20] and other organs (larynx, trachea, vocal cords, supra-renal glands and skin). Clinical symptoms may vary significantly, but weight loss and fever are especially frequent. In general, the pulmonary system (including lungs) is severely affected. Ganglia hyperplasia, hepatomegaly and splenomegaly may also occur, as well as CNS invasion and damage (the condition known as NPCM). In NPCM, aspiration or biopsy of CNS lesions must be avoided due to the invasiveness of neurological procedures, which highlights the need for sensitive and specific immunodiagnostic assays to determine the presence of the fungus in cerebrospinal fluid [21]. Most probably, this scarcely reported condition has been highly underdiagnosed, for which asymptomatic or atypical presentations, unskilled neurological evaluation and lack of suitable and specific diagnostic methods have certainly contributed [11]. Mortality due to NPCM may reach 20% [22], although delayed diagnosis usually worsens this rate. Apart co-infections, differential diagnosis between the two conditions must always be considered as well [14]. The condition of subclinical infection is very frequent in endemic areas, and may affect around 60% of native populations [23]. Moreover, as with tuberculosis and other systemic mycosis, early lesions of PCM may remain asymptomatic for decades until late dissemination [21]. In fact, the latency period of PCM usually lies between some months and several decades. PCM patients frequently exhibit other diseases of either infectious or non-infectious nature, including Hodgkin's disease (and associated carcinomas), tuberculosis (in 5–10% of the cases), enteroparasitosis, chronic pulmonary infections, AIDS and, less frequently, leishmaniosis, leprosy, Chagas disease and other mycosis (*e.g.* histoplasmosis and blastomycosis) [19,24], highlighting the need for accurate differential diagnosis. Among HIV patients, PCM often occurs as an opportunistic infection [9], although at a lesser extent than could be initially expected, probably as a result of its prevalence in rural areas (where HIV cases are less frequent) and to the wide antifungal usage among HIV patients [20]. Nevertheless, increasing cases of PCM–HIV co-infections have been registered, presumably due, at least in part, to the gradual spread of HIV epidemics to small urban centers, close from the rural areas where PCM is endemic. Moreover, it was observed that HIV-positive patients co-infected with the chronic form of PCM usually exhibit clinical-epidemiological patterns that resemble those of the acute form of PCM, being most of these cases due to reactivation of latent foci rather than to recent severe infections [25]. This finding, and the fact that the acute form of PCM usually raises higher antibody levels than the chronic form [26], highlights the importance of differential laboratorial diagnosis and identification of the clinical pattern for adequate follow-up and therapy. Together with the lack of experienced clinicians and the long latent period of the disease, all the aforementioned factors may significantly contribute to the difficulty of diagnosing PCM, especially outside endemic regions, where this disease can be easily misdiagnosed [27]. Ultimately, and given the potential severe and irreversible debilitating effects of this mycosis, rapid and accurate diagnosis is important to promptly begin specific therapy (which is very different, for example, from that for tuberculosis), in order to minimize the risk of the deleterious effects on internal organs [28]. So far, cases of acquired resistance to antifungal treatment in PCM patients have been relatively scarce, but the frequent relapses in chronic infections, by imposing long-termed antibiotic administration, may lead to unfavorable prognosis [29] and, as such, to the future emergence of PCM as an important public health concern.

3. Conventional diagnosis of PCM

Conventional diagnosis of fungal infections usually begins with the identification of clinical symptoms followed by fungal direct examination and isolation from clinical specimens. Laboratorial diagnosis may then proceed by histopathology, which relies on staining of fungal structures for morphological identification. However, unless an experienced observer is in charge, the commonness of atypical morphologies exhibited by many fungi difficult definitive histopathological diagnosis. In non-endemic areas, the etiological agent of PCM may be confused with other dimorphic fungi, such as *Histoplasma* spp., *Blastomyces dermatitidis* or *Coccidioides immitis* [9]. Additionally, histopathology may suffer from lack of sensitivity. In tissues, the presence of cells of different size with various types of sporulation (single blastospores, short chains or multiple budding) can suggest the diagnosis. However, when there is a predominance of small cells, the infection may provide incorrect identification for other fungi [30]. For these reasons, direct examination by microscopy has been considered the reference method for laboratorial diagnosis of PCM and of many other mycosis.

3.1. Direct examination and culture

The "gold-standard" technique for laboratorial diagnosis of fungal infections consists in the direct microscopic examination of clinical specimens and their culturing followed by macroscopic and microscopic observation of the colonies for identification of the fungal species. The identification of a particular fungal disorder has been considered crucial for accurate identification of the etiologic agent and as a marker for active infection as well. In fact, the foremost diagnostic test for PCM involves direct examination of clinical samples for visualizing the causative agent [8]. As an example, microscopy-based distinction between *P. brasiliensis* and *B. dermatitidis* can be relatively easy since, very often, a single yeast cell from *P. brasiliensis* may produce a characteristic multibudding format, while the other yeasts usually produce a single bud [31]. However, accurate microorganism identification through direct examination requires skilled manpower, long times for culture growing and proneness of the target fungus to be cultivated *in vitro*, apart the non-quantitative and the biohazard aspects. In particular, delayed and misleading diagnosis may present several public health concerns, namely the difficulty and lengthiness to initiate and apply appropriate therapy [31,32]. *P. brasiliensis* cultures usually take weeks to grow [20] and must be kept for at least two months before confirming a negative result [8]. As culture features are not differentiated enough for unambiguous identification of the fungus, the suspected colonies should be reverted to the yeast phase for proper identification [33]. This, obviously, severely limits the usefulness of culturing techniques for early diagnosis of infections caused by this agent. This is also a disadvantage when compared to the faster histopathological technique [34]. Another drawback of culturing is that a positive culture may indicate fungal colonization instead of true invasion, especially in the case of opportunistic microorganisms [35]. A general handicap of direct examination techniques is the inability to supply conclusive diagnosis in the case of a negative result since, in this case, the heterogeneity of the examined sample fraction may erroneously lead to assume the inexistence of the fungus in the whole sample. Therefore, it may be desirable and necessary the utilization of alternative laboratorial methods, namely serological or molecular techniques, for improved diagnosis of many fungal infections.

3.2. Immunodiagnosis

Serological techniques are usually simpler than culture and are very useful in the diagnosis and follow-up of patients with this infection. They have suffered considerable advances on the past decades as a result of the development of innovative detection schemes, identification of relevant *P. brasiliensis* antigens [36] and increasing use of monoclonal antibody technology for application in immunoassays [37]. Immunodiagnostic tests for detection of anti-PCM antibodies have been described based on the measurement of soluble reactants (*e.g.* tube precipitation [38], ID [39] and immunoelectrophoresis [40]), cell lysis (*e.g.* CF [41]) or antigen/antibody interactions (*e.g.* latex agglutination [42], erythroimmunoassay [43] and immunoblotting [44]). Among them, ID probably remains the main serological test for PCM, due to its high sensitivity, specificity, simplicity and inexpensiveness [19]. However, enzyme-based immunoassays, usually in ELISA formats, have been increasingly used for serologic diagnosis of PCM and other fungal infections. Although being less specific than ID due to proneness to cross-reactions [21], ELISA is highly sensitive, fast and suitable for high-throughput testing. In general, ELISA is also simpler and cheaper than traditional serological methods for routine laboratory testing. The expected correlation between the seric level of some immunoglobulins and their specific antibody functions against *P. brasiliensis* has been confirmed in some reports, although this seems to be controversial. In some studies, high antibody titres (namely of IgA, IgG and IgE) have been observed in severe and disseminated PCM [19]. There are evidences about competition between different immunoglobulins in serological tests, as is the case of IgE and IgG, being the former usually considered a marker in cases of deep severe mycosis. High IgE levels can be found especially in patients with blastomycosis, coccidioidomycosis and PCM. The classical indirect ELISA (originally developed for detection of IgM), which relies on binding of the solid-phase immobilized antigen to all antibody isotypes proportionally to their concentration in the sample, uses a secondary antibody for detection of specific IgE, but too many false-negative results may arise due to IgG competition for the antigenic sites. Alternatively, in capture ELISA, selective binding of patient IgE to the solid-phase occurs, therefore eliminating IgG competition [45]. The *P. brasiliensis*-specific IgE/total IgE ratio in the sample, measured by capture ELISA, has been claimed to be a more accurate parameter than *P. brasiliensis*-specific IgE only, measured by indirect ELISA [46]. As a general handicap for the diagnosis of infectious agents, serological techniques for antibody detection are of low usefulness in detecting early disease since some days are required to elicit the production of detectable antibody levels. The sensitivity of antibody detection tests may be relatively low in immunocompromised patients, in which only low levels of antibodies circulate in the blood, despite the disease has been, to date, only rarely observed in patients affected by AIDS and other immunosuppressant conditions [47]. A traditional drawback on PCM serology is the potential cross-reactivity between the anti-*P. brasiliensis* antibodies with antigens from other fungi, mainly with *Histoplasma capsulatum* [9], due to the frequent usage of crude preparations of whole microorganisms, containing undefined antigens with high variability. Plus, ELISA studies showed higher reactivity to *P. brasiliensis* antigens in leishmania-seropositive than in leishmania-seronegative dogs. This, although preferentially suggesting co-infection, does not exclude eventual cross-reactivity [48]. In these cases, conclusive serological diagnosis can only be achieved by using paired serum samples (from, respectively, the acute and convalescent phases), but this takes three to four weeks to completion [34]. The quality of the antigens usually varies among different laboratories, depending on the fungal strain, growth phase, culture medium, inocula size, incubation time and recovery techniques [38]. Together with the complexity and lengthiness for production of these preparations [49], this strongly impairs standardization of diagnostic techniques among different laboratories [50]. Compared to antibody detection, the detection of circulating antigens not only allows early diagnosis (crucial to prevent severe symptoms), but also monitoring the effect of antimycotic therapy in fungal clearance from body fluids during treatment [36]. The exocellular gp43 of *P. brasiliensis* is continuously secreted to the yeast cell medium during the exponential growth phase of this fungus [51]; for being found in virtually all isolates [52], it may well be considered the most ubiquitous antigen for serological diagnosis of PCM. In sera of PCM patients, gp43 is frequently found in circulating IgG immunocomplexes [53]. Unlike in acute infections, there are evidences about the occurrence of high seric levels of IgG against polydispersed high molecular mass antigens produced by *P. brasiliensis* in chronic PCM infections [18]. However, the utilization of gp43 for diagnosis may render false-negative results, probably due to gp43 polymorphism [54]. Since the gp43 expression is only roughly characterized [55] and is isolate-dependent [56], a standardized gp43-based serological test for *P. brasiliensis* will require the presence of this antigen in high concentration [10]. Furthermore, the carbohydrate epitopes (which could recognize up to almost 50% of total reactivity of PCM sera) of gp43 may cause some cross-reactivity in cases of histoplasmosis or lobomycosis [57]. This is highly related to the fact of galactose

being the main common epitope among pathogenic fungi. There have been attempts to eliminate the cross-reactive carbohydrate epitopes of gp43, including those responsible for the intense cross-reactivity of *P. brasiliensis* with *H. capsulatum*, but they have not been well succeeded towards the development of a specific ELISA test for PCM [58]. In addition, the methods for detection of circulating antigens may suffer from low sensitivity [59] and strongly depend on the sourced biological fluid which, apart serum, can be bronchoalveolar lavage fluid (in the case of pulmonary infections) [60] and cerebrospinal fluid (in the case of CNS infections) [61]. Thus, gp43-related antibodies can be used as viable alternatives for routine testing [62], as a strong and long-lasting antibody response against the gp43 antigen is usually observed in PCM patients [63]. In fact, 90% of PCM patients present positive results for gp43-based ID assays and around 100% in immunoblotting assays [64]. The p27 has been considered a suitable alternative for serological diagnosis of the fungus. This recombinant antigen has shown to be reactive against a high proportion of patient sera with PCM [65], reaching considerable sensitivity and specificity in the ELISA test format [66]. Taking into consideration the relative expensiveness for producing the gp43 and complexity in the case of the p27 (another relevant antigen of *P. brasiliensis*), a fast and inexpensive assay for diagnosis of PCM with subcloning and expression of the p27 gene was reported, in a dot-blot test format where the recombinant protein, produced in high amount, was used as the antigen [28]. Excellent sensitivity and specificity (both around 100%) were claimed, and the dot-blot immunoassay format showed to be a viable alternative to ELISA. The authors also claim better performance than that obtained in a similar work employing gp43 [67], probably due to the use of a recombinant antigen rather than a purified one. In this last study, gp43 was treated with sodium metaperiodate to avoid cross-reactivity with histoplasmosis serum. Besides gp43 and p27, other antigens have been used for PCM diagnosis, namely the gp70 [36], the hsp60 [68] and the hsp87 [69]. Environment-related conditions (*e.g.* climate, soil type and pH, temperature and humidity) may influence the immunogenic patterns of *P. brasiliensis* isolated in different regions [10], but the geographical origin of the isolates itself may also play an important role [1]. Indeed, the region of the Mato Grosso State, in Central-Western Brazil, has a high incidence and daily reports of PCM and forms a distinct geographic region for the acquisition of the disease [70]. So far, the Central-Western region of Brazil is the only where the *P. brasiliensis* Pb01-like isolate cluster (by reference to the previously known Pb01 isolate) was isolated [71]. This cluster shows significant genetic divergence with the other *P. brasiliensis* isolates [72], in such an extent that it has recently been reclassified as a new species (*Paracoccidiodes lutzii*) within the *Paracoccidioides* genus [73]. This may have significant implications for serological diagnosis of PCM in that endemic region. In fact, several confirmed cases of PCM, especially originated from that region, have exhibited seronegativity. Such false-negatives are probably due to the fact that antigens for serological tests are usually produced from species from the *P. brasiliensis* S1 group (of one the most abundant, existing in other South-American countries [71]), as confirmed by ID-based studies showing the importance of the differences in antigenic composition for unambiguous PCM serological diagnosis [1]. Many studies have also evidenced significant differences in results for the same PCM serum when distinct serological techniques are employed. The variable specificity of immunodiagnostic tests suggests the need for establishment and standardization of a single technique within each clinical setting [38]. Alternatively, as a result of the production of well-defined and reproducible antigens permitted by the unending advances in molecular biology, immunodominant antigens for PCM serology can now be obtained by cloning and expression. By using standard recombinant antigens, cross-reactions can be minimized in relation to the use of crude antigens [28]. Nevertheless, cloning and expression of antigens is still hampered by the complexity of the process and high costs. In short, despite its usual limitations, the advantages and versatility of serology, which continues to evolve in a great number of formats, either for antibody (*e.g.* by latex bead agglutination [26]) or antigen (*e.g.* by inh-ELISA [34,52,60,63]) detection, have proven undoubted value for routine laboratorial diagnosis of PCM infections.

4. Molecular diagnosis

The advent of molecular diagnosis has brought a new and improved range of essentially quantitative techniques. Molecular assays have evidenced success in detecting *P. brasiliensis* even when microscopic observation and antibody detection fail [32]. PCR, in particular, has shown greater usefulness when antigen or antibody blood levels are too low for successful immunodiagnosis [74]. In general, quantitative methods for detection and identification of human pathogenic fungi exhibit better performances, especially in terms of specificity, than qualitative methods. This strongly enhances their ability to predict invasive fungal disease [75]. Unlike traditional diagnostic assays, molecular methods will undoubtedly continue to be useful in rapidly detecting invasive fungal infections with high sensitivity.

4.1. PCR

As far as it is currently known, *P. brasiliensis* consists in a species complex with distinct genetic groups. Thus, molecular methods are essential to distinguish and identify circulating genotypes of the fungus [6]. Molecular diagnosis of fungal infections usually relies on PCR and other PCR-based techniques for amplification of fungal genomes, whose blood levels are usually too low for successful detection without a previous amplification step. Conventional PCR does not yield quantitative results, which are important to evaluate disease progression. Thus, quantitative methods (*e.g.* culturing) are usually required for downstream analysis in order to assess the effect of the fungus and to decide whether to treat, or not, the fungal disease. In parallel, automated DNA synthesis is a relatively recent achievement. The resulting production of molecular probes with consistent and defined properties has improved the reproducibility, sensitivity and specificity of fungi diagnosis [34]. Nested-PCR, which uses a second set of primers (internal to the first, original primer set) to amplify, in a second amplification round, an internal and thus very specific genomic region, is not only inherently more specific but also more sensitive than conventional PCR. On the other hand, RT-PCR, by using the reverse-transcriptase enzyme before the PCR step, allows the amplification of fungal RNA by intermediate synthesis of cDNA, which can then be used as a conventional PCR template. Real-time PCR is a highly sensitive and quantitative PCR-related technique integrating standard PCR and detection with fluorescent probes in the same reaction vessel. Emitted fluorescence can then be quantified and correlated with the amount of PCR amplicons. Unlike in standard PCR, the accumulation of PCR products is automatically screened and recorded after each amplification cycle by using fluorescent DNA intercalating dyes (*e.g.* SYBR Green I), sequence-specific probes [76] or molecular beacons [77]. Intercalating dyes are usually cheaper but have low selectivity, since they bind every double-stranded DNA molecules, irrespectively to their sequences. This, of course, does not happen with the use of sequence-specific probes, which further allow detection of SNPs. Furthermore, the possibility of tagging probes with different reporter dyes allows monitoring simultaneously different fungal isolates for better assessment of interaction between different isolates and population analysis. Real-time PCR can also be useful to distinguish between simple colonization, subclinical

infections and actual disease caused by fungus. Compared to PCR and other PCR-based methods, real-time PCR exhibits, in general, similar sensitivity (usually higher than that of culturing methods, especially in the case of slow-growth pathogenic fungi, like *P. brasiliensis*) and specificity, decreased assay time (by precluding the need for a post-amplification processing and detection), and reduced environmental contamination (since amplification and detection take place in the same closed reaction vessel). This is extremely relevant as a consequence of the ubiquitous presence of many environmental fungi [31]. This may be especially important in fluorescent *in situ* PCR, a technique that employs fluorescently labeled genomic probes coupled to detection by confocal microscopy, allowing detection of non-cultivable microorganisms and of fungal/host interactions, as well as quantitative detection of fungal PCR amplicons in fixed environmental samples [78]. The successful exploitation of PCR-related techniques for microorganism identification strongly depends on the chosen DNA extraction protocol (which may have to account for the eventual need for rapid extraction, for high DNA purity or for the type of environmental source) and on the selection of target oligo- or polynucleotide sequences. This last feature can be carried out by cloning and sequencing arbitrary portions of fungal genomes, or by selecting specific sequence information from online DNA databases in order to design PCR primers to target conserved or variable genomic regions. Fungal sequence analysis has been considered less robust than for bacteria due to the endless changes in fungal taxonomy, to the exiguity of current fungal sequence databases and to the inexistence of a wide conserved genomic region for fungal identification at the genus or species level [79]. Moreover, sequencing methods for identification of filamentous and dimorphic fungus (as is the case of *P. brasiliensis*) have not been as extensively evaluated as those for yeasts. Pyrosequencing technology, originally developed by Biotage AB (Sweden), is an adaptation of the classical Sanger dideoxy sequencing method in which inorganic phosphate released from a DNA chain growing by the action of a DNA polymerase produces measurable light by the enzymatic activity of sulfurylase and luciferase [31]. The technique is faster and cheaper than conventional sequencing, but is still limited to the analysis of short templates (around 100 bp), which hinderers good discrimination among closely related fungal species [80]. Criticism about molecular diagnostics usually relies on the fact that positive-tested samples often correspond to simple colonization or to latent infections rather than to current infections, and that, even among these, not all lead to disease [81]. The absence of more systematic evaluation and standardization of PCR-based molecular diagnostic tests for fungal disorders has been a major constraint, but initiatives like the UKPCG [82] may ultimately pave the way for a wider implementation of these tests into laboratorial routine testing.

4.2. Primers and probes for molecular diagnosis of P. brasiliensis

Concerning specific-primer design for molecular diagnosis of fungal species, highly conserved regions acting as primer binding sites must exist within the target gene, and these regions must flank regions with enough sequence variability to permit genus- or species-level discrimination. Fungal ribosome genes, especially those related with 18S RNA, exist in all organisms in high amounts, which favors high sensitive detection [9]. The rRNA gene contains both conserved (28S, 5.8S and 18S) and specific (ITS1 and ITS2) regions, turning it valuable for fungal identification [34]. Accordingly, high-rate mutation regions are useful for discrimination among different genus or upper phylogenetic levels, while low-rate mutation regions are especially useful for species differentiation [6]. In addition, around 100 copies of this gene exist in the corresponding chromosome, which constitutes a suitable pre-amplification step for subsequent PCR amplification [83]. The genes of rDNA are among the most commonly targeted sequences for detection of *P. brasiliensis* in clinical samples. A work reporting the targeting of the 5.8S and 28S rDNA genes with fungal universal primers followed by more specific primers (OL3 and UNI-R) for nested-PCR resulted in good discrimination of *P. brasiliensis* from human DNA and other pathogenic fungi, including *H. capsulatum* [74]. Nested-PCR was also employed for highly sensitive and specific detection of the fungus and strain identification by means of the PbITS1s and PbITS3a set of primers [84]. These primers amplified specifically a 418 bp DNA fragment in the ITS regions. In a recent work, a labeled molecular beacon probe was directed to the ITS1 region of *P. brasiliensis* rDNA, with highly reproducible, specific and sensitive real-time fluorescent detection [85]. Another work developed a method of sequential utilization of universal fungal primers to target corresponding conserved regions of rDNA from several yeast-like fungi, including *P. brasiliensis*, followed by fungal specific primers for fungi identification at the species level. Detection was carried out by colorimetric ELISA, which, unlike amplicon size-based detection (*e.g.* after conventional electrophoresis and ethidium bromide staining), prevents erroneous fungal diagnosis due to the fact that different fungi may produce characteristic amplicons with the same size. This PCR/ELISA detection method, which is also simpler to perform than Southern-blot, may be used for fungal identification in culture and in clinical samples as well. The technique proved to be useful in differentiating the studied fungi species and suggests the possibility of designing an array for simultaneous detection of different fungi after a single PCR amplification step followed by the use of specific probes. The sensitivity of this PCR-ELISA test may be further improved by using, for instance, nested-PCR or new forms of *Taq* polymerase, sufficiently stable to stand more PCR cycles [34]. Experiments with the 18S rRNA genes of *P. brasiliensis* evidenced high homology between this fungus and *B. dermatitidis* and *H. capsulatum*, which has often motivated the use of the gp43 associated gene to enhance the specificity of PCM diagnosis [57]. After full cloning, sequencing and characterization of the gp43 gene coding region [52], several primer sets have been designed for molecular diagnosis of *P. brasiliensis*. These genes have been widely used to specifically target *P. brasiliensis* genome sequences in biological samples [86]. DNA-based vaccination against *P. brasiliensis* with the gp43 gene has also been successfully assayed in mice [87]. Works employing sensitive nested-PCR [9] and fluorescent real-time PCR [88] detection have also been reported. The gene of the 27 kDa antigenic protein has also been used for detection of *P. brasiliensis* in wild animals, inoculated mice and artificially contaminated soil [89]. Other works have described the development of molecular assays based on the 0.72 and/or on the 0.83 kbp DNA fragment, which are common to all *P. brasiliensis* strains [32,90]. In addition to clinical diagnosis, *P. brasiliensis* epidemiology and phylogeny can also benefit from PCR-based technologies. PCR studies where specific primers designed to target the 43 kDa and the 27 kDa protein genes of *P. brasiliensis* were used, showing the possibility of successfully isolating this and other environmental microorganisms from the soil and hence contributing to identify their habitats [91]. Conventional culture and animal inoculation techniques usually exhibit low reproducibility in this regard [10]. This is due to the presence, in soil samples, of natural contaminants (*e.g.* polysaccharides and humic acids) that strongly interfere with the PCR reaction, as a consequence of their resemblance with DNA in terms of chemical nature and solubility [92]. Aiming to investigate a possible correlation between certain characteristics of *P. brasiliensis* isolates and virulence in humans, PCR was used to amplify DNA fragments containing microsatellites as molecular markers to discriminate various *P. brasiliensis* isolates and relate them with different pathogenicity patterns [91]. In particular, it was also observed that gp43 gene sequences of

some *P. brasiliensis* isolates were phylogenetically distant from the sequences of other isolates, suggesting a possible correlation between some *P. brasiliensis* gp43 gene polymorphism and the pathogenic degree of these strains [93]. While some works (for example, employing RAPD analysis) have confirmed this correlation [94], the above mentioned study with microsatellites did not. Other authors working with RAPD suggest that, instead of being correlated with clinical manifestations, genetic differentiation can be associated with the geographic regions of endemicity [95]. The microsatellite study also showed the possibility of using the p27 gene to screen *P. brasiliensis* in known natural reservoirs (animals and soil), although reports about its utilization in human samples have not been found so far in the literature [89]. The RAPD technique has recently been used to detect and characterize, for the first time, genetically distinct isolates of *P. Brasiliensis* from a single patient, by using different random primers [96]. The significant genetic variability thus detected, above 28%, confirms the genetic complexity within this fungal species and opens a new road for clinical studies.

4.3. Commercial diagnostic kits

The AccuProbes, from Gen Probe (San Diego/CA, USA), are among the few commercially available molecular tests for fungal analysis. These kits usually rely on specific nucleic-acid hybridization followed by chemiluminescent detection and allow highly sensitive and specific identification of dimorphic fungus in culture [97]. After cell lysis and DNA extraction, sequence-specific hybridization between a chemiluminescent single-stranded DNA probe and the complementary rRNA from the target organism occurs. Since a PCR step to amplify the target is not used, fungal growth in pure cultures is usually required to achieve the reported sensitivities. In a study about the evaluation of AccuProbe assays for rapid identification of *B. dermatitidis* and *C. immitis*, the inclusion of *P. brasiliensis* isolates for comparison showed, once more, the morphological and antigenic relatedness of *P. brasiliensis* with other fungal species [98]. Although *P. brasiliensis* endemic regions (in South America) are different from those of *B. dermatitidis* (in North America), imported cases in USA and Europe among immigrants [27] have raised concern about the inability of the *B. dermatitidis* probe for conclusive diagnosis in such cases [99]. Some works have reported other fungal hybridization probe-based systems, although not commercialized yet or even routinely used [34]. A common problem of many commercial kits for disease diagnosis is the relatively long assay time and tedious sample processing, making difficult to considerer them as true and valuable rapid diagnostic tests for decentralized, point-of-care applications [100].

4.4. Microarrays

Microarray technology may constitute a step forward towards the development of high-throughput detection systems for bioanalysis and assessment of gene expression patterns, due to its ability for multiplexed qualitative and quantitative testing of very small volumes of complex environmental samples containing hundreds or thousands of different species [101]. Unlike single-analyte tests, microarrays can offer multi-component information and dynamic compensation for sample matrix effects [102]. The utilization of microarrays for fungal diagnosis has already been reported [103,104]. Genomic microarrays have already been employed for the identification, *in vitro*, of genes and gene expression during growth of *P. brasiliensis*, in which the distinct expression patterns observed between the yeast and the mycelium form corroborate the well known physiological features of both forms [105]. The ability of microarrays for simultaneous scanning of multiple pathogens constitutes, in general, a significant advantage, in terms of high-throughput and cost-effectiveness per assay, to current single-analyte tests [31], usually based on PCR. This has been made possible with high-density microarrays, although further improvements are still required to manually handle and to analyze biological samples with millions of different genes without simultaneously increasing contamination, the number of false-positive results and the background signal [78]. The ability of optical devices to carry huge amounts of information may suitably explain the high commercial success of optical transduction-based microarrays compared to electrical ones [81]. However, common drawbacks include difficulty of scaling down the array density, limited resolution, high variability of the response signal due to sample concentration and dependence on a prior biomolecule amplification step [106,107]. Further evaluation and standardization of microarray techniques for fungal detection and identification are still required before they can be widely implemented in laboratorial routine analysis [31], as well as lower costs for the current expensive and complex equipments required. In general, these technologies still do not accomplish the full criteria for in-the-field (*in situ*) applicability, seeming rather laboratory-based detection systems. There is an urgent need to develop new and improved diagnostic tests able to provide sensitive, specific, reproducible, simple and cheap detection of invasive and systemic pathogenic fungi, both endemic and opportunistic, for diagnosis within healthcare settings and for in-the-field applications as well.

Table 1 summarizes the main operational features from most of the techniques and methods referenced in this chapter concerning the laboratorial diagnosis of *P. brasiliensis*. This table does not intend to be exhaustive, but rather to pinpoint some quantitative landmarks that, ultimately, improved diagnostic tests for this fungus will face in the future. The recent search for new rapid diagnostic tests with improved sensitivity and specificity has driven the application of molecular diagnostic techniques to PCM in only a reduced number of patients and experimental models, within some laboratories, in order to complement routine classical diagnosis. These are mainly in-house methodologies without proper standardization and, therefore, (still) unpublished. There is still the need to optimize and standardize DNA extraction from different clinical samples and to solve other common handicaps and challenges of molecular detection methods. Owing to these constraints, as well as to the recurrent lack of improved equipment and skilled manpower in low-income settings and regions where PCM is endemic, it will certainly take a long time until molecular techniques become more widely implemented to empower routine laboratorial diagnosis of this illness.

5. Biosensors for fungi

Biosensor technology is expected to provide suitable methodologies for fungal detection and diagnosis of mycosis. In biosensors, the biorecognition event occurs directly on the surface of a physical transducer and, unlike in conventional bioassays and bioanalytical devices, the assay design is permanently fixed on the device platform. Biosensors are usually designed to be highly sensitive, selective, fast, portable, cheap, simple and, in general, response-proportional. Moreover, when dealing with pathogens, disposability is usually preferred to reusability. Additionally, biosensors for label-free, real-time and *in situ* detection will certainly enhance the ability to detect microorganisms in different environments. Compared to microarrays, biosensors do not require surface scanning for acquisition of full information in order to yield an output. In parallel, nanotechnology has rendered a multitude of new analytical procedures, structures and systems for biosensing, as a consequence of the inherent small size and unusual physicochemical properties of nanoparticles. The convenient application

Table 1
Summary of the main characteristics of some laboratorial techniques for diagnosis of *P. brasiliensis* described in the literature.

Type of test	Biotarget	Bioprobe	Reported performances	Reference (year)
Immunologic (agglutination)	Serum antibodies	Latex (polystyrene) particle-attached fungal cell antigens	Sensitivity: 60.8–69.5%	[54] (1978)
Immunologic (competitive ELISA)	gp43	anti-gp43 antibody	6 ng ml^{-1}	[47] (1992)
Immunologic (indirect ELISA)	Serum antibodies	Recombinant p27 antigen	73.4% sensitivity and 87.5% specificity	[80] (1998)
Molecular (PCR)	p27 and gp43 genes	3 specific primer pairs	LOD of 3 pg (90 cells)	[100] (1999)
Molecular (nested PCR)	gp43 gene	2 primer pairs	LOD of 0.5 fg (~100 gene copies)	[40] (2000)
Molecular (modified PCR–ELISA)	rRNA pan-fungal gene	Biotinylated universal and *P. brasiliensis*-specific primer pairs	LOD of 3.2 pg	[52] (2001)
Immunologic (immunoblotting)	Serum antibodies	Recombinant HSP60 protein	97.3% sensitivity and 92.5% specificity	[80] (2002)
Molecular (real-time PCR)	Sequence from the gp43 gene	Fluorescently labeled primer pair	LOD up to 10 molecules of DNA per reaction	[99] (2002)
Immunologic (inh-ELISA)	gp70	Specific monoclonal antibodies	100% sensitivity and 100% specificity	[82] (2004)
Molecular (PCR)	Universal gene sequence	Specific primers	10 pg	[101] (2005)
Immunologic (dot-blot assay)	Serum antibodies	Recombinant p27	100% sensitivity and 98% specificity	[43] (2006)
Immunologic (ID)	Serum antibodies	Exoantigen 550B (Ag 550B)	92.3% sensitivity and 98.4% specificity	[8] (2008)
Molecular (real-time PCR)	DNA sequence from the ITS1 region of rDNA	Labeled molecular beacon	LOD of 1 fg μl^{-1} and sensitivity of 100% (respiratory samples)	[95] (2009)

LOD, limit of detection.

of nanotechnological tools to the biosensor field has been increasingly exploited as an emerging theme within nanobiotechnology, to produce improved and innovative schemes and devices envisaging disease diagnosis.

5.1. Mass-sensitive and electrochemical biosensing for fungi

A mass-sensitive biosensor to detect the growth phase of *Aspergillus niger* and *Saccharomces cerevisiae* (as models for the behavior of pathogenic fungi) with gold-coated and uncoated silicon microcantilevers functionalized with fungal surface-interacting proteins was developed [108]. Spore immobilization led to a specific resonance frequency shift, reaching a detection limit of 10^3 CFU ml^{-1} and assay time of only 4 h, unlike the several days required by conventional techniques. One of the few reported biosensors employing living microbial cells as templates for MIPs was developed [109]. It relies on the QCM principle for whole yeast cell detection and on the well-known sedimentation behavior of yeasts [110] (Fig. 1). A detection limit of 10^4 cells ml^{-1} was reached in complex media. The QCM and other types of mass-sensitive biosensors have been less exploited than optical and electrochemical ones. This is caused by the fact that their performance and sensitivity strongly depend on environmental conditions, even very small fluctuations in air flow and humidity. Too often, washing and subsequent drying steps must be meticulous to obtain rigorous measurements. In addition, interfacial phenomena between the outer air phase and the bioanalytical liquid medium strongly impair accurate quantitative predictions. Understandably, this severely limits their usefulness for in-the-field applications. On the other hand, electrochemical transduction seems to be appropriate for production of simple, inexpensive and miniaturized (portable) devices [81]. An alkanethiolate SAM/gold-based impedimetric biosensor for detection and pioneer quantification of living yeast cells (from *S. cerevisiae*) immobilized onto an electrode surface was reported [111], whereas, a similar aminealkanethiol SAM-based immobilization procedure had already been assayed for detection of yeast DNA hybridization by voltammetry [112]. This technique was also employed for PCR-free detection of *Trichoderma harzianum* species-related oligonucleotide probes at a gold disk electrode [113]. In another work, monoclonal antibodies against *Candida albicans* were adsorbed onto SWCNTs, thus providing specific binding sites for fungal antigens [114]. The detection mechanism relied on a FET with the SWCNT network as the conducting channel (Fig. 2). The resulting variation in the electrical conductance is probably due to a physical distortion of CNTs upon the biorecognition event or to charge-transfer [115]. Although exhibiting some cross-reactivity between anti-*Candida* antibodies and existing *S. cerevisiae* cells, the sensor was able to detect as few as 50 CFU ml^{-1} of yeast cells (in only 1 h), nearly half of the detection limit of current detection methods. This is, most likely, the first application of CNT-based FETs to the detection of eukaryotic cells. In general, the main handicaps of electrochemical biosensors are the propensity for interferences caused by electrochemically active compounds in the response signal and electron-transfer blocking effects between electrodes and sensing layers [81].

5.2. Optical biosensing for P. brasiliensis

Until recently, there have been no reports about the application of biosensors or nanotechnology to the detection of *P. brasiliensis*, but an ongoing financed project in Brazil may be, most likely, the pioneer in this endeavor [116]. The project concerns the application of *P. brasiliensis*-related DNA nanoprobes linked to gold nanoparticles and testing of clinical fluid samples aiming to target the complementary DNA chains. After fungal DNA extraction and amplification, detection will be carried out by optical methods. Compared to electrochemical transduction, optical transducers, in general, are still too complex, expensive and less amenable for miniaturization; however, the ability of optical signals to carry enormous amounts of information in a very fast and safer way, apart the easier sterilization, make them more biocompatible and thus more promising for ultimate *in vivo* biosensing, namely by making use of fiber optic technology [81]. The methodology proposed in the Brazilian project lies among the most modern and innovative approaches for disease diagnosis. It is expected that, in a short term, successful

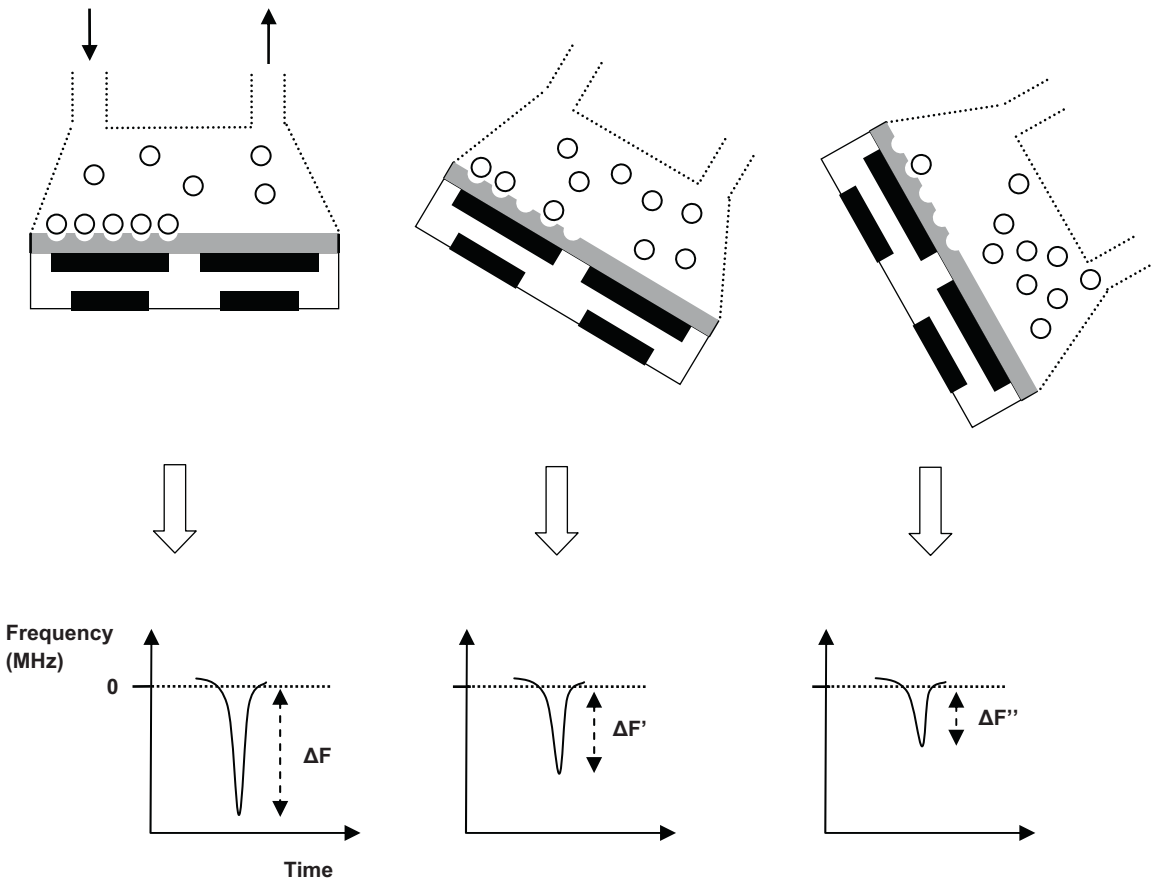

Fig. 1. Rotating QCM for yeast cell detection. Two major electrodes (upper black boxes), embedded in the QCM (white box), were coated with a polymer (gray box), while the minor electrodes (lower black boxes) faced downward. One of the coated electrodes was surface-imprinted with whole yeast cells, thus allowing differential measurements by comparison with the non-imprinted (reference) electrode. This layout was assembled within a flow cell (dashed cage, with arrows representing the inlet and outlet), where yeast cells (white circles) circulate. Most of these cells adhere to the tailored, imprinted cavities. Rotation of the whole setup towards the vertical position results in a gradual decrease of the response signal (frequency shift, represented, respectively, by ΔF, $\Delta F'$ and $\Delta F''$), due to yeast cell disassembly and sedimentation. Along the process and under the tested conditions (*e.g.* of flow rate), cell adhesion to the reference electrode was negligible.

results may be obtained and published in scientific broadcasting regular media. In addition, this also constitutes a rare reported application of optical methods for detection and identification of fungi.

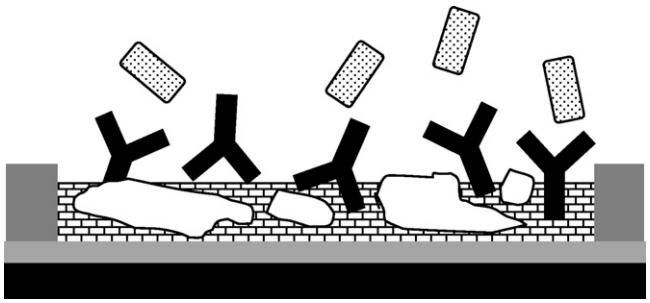

Fig. 2. CNT-based FET biosensor for detection of yeast cells. The CNT network (bricked wall-like box) was directly adsorbed onto a SiO_2 layer (light-gray box) and this, in turn, onto a Si layer (black box). The source and drain (dark-gray boxes) of electric current were placed in the extremities of the assembly. Specific monoclonal antibodies (Y-shaped black boxes) were then immobilized onto the CNTs and a blocking reagent (white spots) used to cover the unprotected gaps of the CNT walls, thus preventing non-specific binding of other yeasts or proteins. Exposure to a biological sample containing the target yeast cells (dotted rectangles) causes a significant decrease of the CNT conductance as a result of antibody–yeast cell binding. This may be due to CNT physical distortion or to enhancement of charge-transfer to the CNTs upon binding of the biological target.

6. The future of fungal diagnosis: final remarks

There is still a long way to go from well-standardized molecular methods and high-throughput microarrays to the newest biosensor technologies, especially in the form of rapid tests for simple, rapid, inexpensive and *in situ* diagnosis. Technological platforms based on lateral flow immunochromatography, molecular imprinting, surface plasmon resonance, fluorescence polarization and electronic noses – frequently in tight relation to bionanotechnological improvements – are just now being applied to the detection of mycotoxins and mycotoxigenic fungi [117], although only scarce reports have been found for non-mycotoxigenic, yeast-like fungi, and none for *P. brasiliensis*. For the identification of mycotoxigenic fungi, specific mycotoxins for protein-based detection and mycotoxin-producing genes for DNA-based detection may be suitable biomarkers. For the remaining fungi, including *P. brasiliensis*, biomolecular detection may eventually be more difficult, aggravated by the multi-cellular and large-dimension features of most of these organisms, compared to bacteria or virus. DNA-based diagnosis, in particular, usually requires prior DNA extraction from cells, a significant obstacle for easy fungal detection due to the common thick and rigid cell walls of fungal species. Compared to the transducing mechanism, the sample pretreatment step has been considerably underestimated in biosensor development, which, obviously, constitutes a further limitation in the case of fungal diagnosis (especially mycelia-forming fungi). A good new in this regard is that very efficient kits for automatized nucleic-acid extraction

from fungi cells are already available commercially. In the near future, these systems may ultimately be integrated in lab-on-a-chip and microfluidic platforms with modules for sample detection coupled to upstream biological sample processing. Interestingly, as in bacteria, yeast cell surfaces are, very often, negatively charged at physiological pH values [118], which may favor the design of electrochemical biosensing mechanisms, namely through binding to charged polymers. In fact, this characteristic may largely explain the predominance of reported electrochemical sensors for fungi compared to other physical transduction mechanisms, as seen above. In most cases, as for other eukaryotic organisms, whole-cell detection has been employed in biosensing layouts. Rather than a possible side-effect of the very small number of reported biosensors for fungi compared to those for other microorganisms, *e.g.* bacteria and virus, this may be seen as a result of some advantageous characteristics of fungal cells. Here are included the possibility of responding to a wider number of substrates from fungal enzymes and the high stability of fungal cells in the sensor environment, resulting from their high tolerance to large variations in pH, osmotic pressure and temperature (leading to increased shelf-life) [119]. Difficulties and challenges will persist to develop autonomous commercially available rapid tests for disease diagnosis from simply proof-of-concept biosensors [100]. Some of these challenges have been extensively reviewed in the literature [81], but of note is that, more than the lack of solid scientific knowledge, the development and commercial exploitation of standardized rapid tests may have been hindered by cost/benefit considerations, especially when mass-production and high-throughput testing are envisaged [120]. Nevertheless, in the future, we may foresee the development of improved sample processing platforms coupled to highly sensitive detection schemes, probably in the form of lab-on-a-chip and/or microfluidic devices, for economically attractive laboratorial and in-the-field diagnosis of *P. brasiliensis* and other systemic and opportunistic fungi.

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