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Non-culture based diagnostic tests for mycotic infections

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Non-culture methods being developed and evaluated for mycotic infections include polymerase chain reaction (PCR), galactomannan (GM) antigenemia, Western blot (WB) to detect antibodies, and detection of the fungal metabolites D-arabinitol and (1,3)-β-D-glucan. Sample preparation for PCR from blood specimens depends on fractionation of peripheral blood, its pre-incubation in blood culture broth, or a total DNA method, which does not rely on fractionation, or pre-incubation. Targets for PCR of fungi in the 18S or ITS2 subunits of the ribosomal RNA genes facilitated the design of *Aspergillus* and *Candida* genus and species probes. Amplicons were identified using PCR-enzyme linked immunosorbent assay (ELISA) or reverse line-blot formats. A pilot study indicated that PCR tests on blood specimens were positive at least once in patients with confirmed invasive aspergillosis (IA). When serum-PCR and serum-GM tests were compared in IA patients, antigenemia was more often positive. PCR detected *Aspergillus* DNA in bronchoalveolar lavage specimens from patients at risk even when cultures were negative. D-Arabinitol can be detected as a marker of candidiasis with gas chromatography-mass spectrometry or enzyme dependent-fluorometry. Each method can differentiate the microbial D- and host L-enantiomers. (1,3)-β-D-Glucan is produced by most genera of pathogenic fungi and can be detected in plasma by the 'G-test'. In patients with febrile neutropenia the efficacy of azole therapy correlated with plasma (1,3)-β-D-glucan concentrations of ≥ 10 pg ml^{-1}. The diagnosis of early acute pulmonary histoplasmosis can be improved by a WB test utilizing deglycosylated M antigen, a 94-kDa glycoprotein. The identity of M antigen as a catalase was deduced from the sequence of the cloned gene. PCR identification of *Histoplasma capsulatum* cultures was accomplished with primer pairs selected from H and M antigen gene sequences.

Keywords fungal PCR, galactomannan, *Histoplasma*, M antigen

Introduction

A broad range of serological tests have been developed for invasive fungal infections [1] but a new generation of more rapid, sensitive, non-culture diagnostic tests are needed. This need encompasses both kits to use in local laboratories and tests to be performed in regional or reference laboratories where there are advantages of economy of scale or specialized technology is available. Non-culture tests are needed to make a timely diagnosis of invasive fungal infections and to select appropriate therapy. The rising number of these infections in devel-

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oped countries is directly related to the number of iatrogenically compromised hosts who are at greatest risk for invasive aspergillosis (IA) and invasive candidiasis (IC) [2]. The worldwide epidemic of acquired immune deficiency syndrome (AIDS) continues to bring an increasing burden of fungal disease, including the endemic mycoses [3]. Wider therapeutic choices make a specific diagnosis more important to establish [4]. Moreover, prophylactic treatment of patients at risk is controversial because of concern for the development of resistance to azole antifungal drugs [5]. Against this background, gradual progress has been made in advancing the detection of fungal antigens, DNA and metabolites. Frequently, such tests receive a favorable clinical evaluation only to fall short of reaching the commercial marketplace. This article is, therefore, an effort to chart progress with more promising non-culture methods, most of which are being evaluated and moving towards commercial development and wider availability. Several of the methods and tests reviewed here have not received a sufficient evaluation to warrant their use as indications to guide clinical treatment decisions. In that respect, this article is positioned near the forward edge of test development and evaluation.

In the first segment three non-culture methods will be discussed: polymerase chain reaction (PCR) for *Candida* and *Aspergillus* species from peripheral blood specimens, galactomannan (GM) antigenemia detection in IA, and the utility of detecting the 5 carbon polyol, D-arabinitol (DA), as a marker of IC. Following that segment is a more detailed discussion of PCR approaches to the diagnosis of IA and IC including a total DNA method for sample preparation from whole blood and the utility of a reverse line-probe assay for IC. Next, another non-immunological approach to diagnosis discusses the detection of plasma (1,3)-β-D-glucan in invasive fungal infections. Finally, the immunodiagnosis of a primary systemic mycosis, histoplasmosis, is discussed highlighting the early diagnosis of acute pulmonary histoplasmosis by detecting anti-H and anti-M antibodies using Western blotting (WB), a dot immunobinding assay, and a consideration of molecular identification of *Histoplasma capsulatum*. For a further discussion of molecular and immunodiagnostic methods for mycotic diseases, the reviews of Reiss *et al*. [1] and Walsh *et al*. [6] are recommended.

PCR for *Candida* species and *Aspergillus* species from peripheral blood specimens

Candida albicans and related *Candida* species are cleared from the peripheral blood by antibody and non-antibody dependent receptor-ligand interactions, affecting the yield from direct culture methods. Blood cultures may be positive in only 58% of IC cases [7]. Quantitative blood cultures indicated that 26·5% of all positive cultures studied contained <1 cfu ml^{-1} of blood; and an additional 27·9% had 1–10 cfu ml^{-1} [8]. Given the low number of cfu ml^{-1} and the refractory fungal cell wall, sample preparation from whole blood has been a challenge to the development of a clinical PCR test.

Efficient sample preparation is also important in IA because, although *Aspergillus* species are angioinvasive, positive blood cultures are uncommon, and are recovered in $\leq 10\%$ of cases. Moreover, glycanases, which efficiently produce spheroplasts from *Candida* species, such as zymolyase, are inefficient with respect to *Aspergillus* species. To overcome this obstacle, sample preparation includes a hot dilute alkali extraction step [9] or uses lyticase, an alternative source of (1,3)-β-D-glucanase [10].

If blood cultures do not provide a high sensitivity for detecting *Candida* and *Aspergillus* species infections, it may seem a contradiction to utilize a PCR approach to detect those fungi in blood specimens. Encouraging reports of detecting *Aspergillus* and *Candida* by PCR methods from blood specimens suggest that small populations of cfu, non-viable fungal elements, or extracellular DNA released as a result of phagocytosis are amenable to PCR amplification. Earlier reports of PCR-based methods to detect fungi in blood were reviewed in [11]. Recent reports include [9,10,12,13].

Towards a standard protocol for Aspergillus *and* Candida *spp. PCR from blood specimens*

There have been many reports on applications of PCR for detecting fungi or fungal DNA from blood or serum and it is not within our scope to review them here. Instead, elements of a standard approach can be recommended for the three major phases of clinical PCR assays for IA and IC: Sample preparation; target and probe selection, and PCR and post-PCR analysis.

Sample preparation

Whole blood specimens may be worked up from anticoagulated peripheral blood, from blood processed using a commercial kit, or by short-term incubation of blood samples in blood culture broth bottles. Later in this article, an alternative method will be described in which fungal DNA is extracted from whole peripheral blood without selective lysis of leukocytes, erythrocytes, and avoiding the necessity for cell wall lytic enzymes.

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Whole peripheral blood

This uses the method described previously [9]. Whole anticoagulated blood, 3–5 ml, is treated to lyse erythrocytes and leukocytes, and the sediment, containing any fungal elements, is collected by centrifugation. Next, the sediment is briefly boiled in dilute alkali to permeabilize walls of filamentous fungi. Efficient spheroplasting of *C. albicans* and non-*C. albicans Candida* spp. is accomplished with zymolyase, including EDTA to chelate divalent cations, which play a role in wall integrity, and 2-mercaptoethanol, to reduce disulfide bonds. Lyticase, another source of (1,3)-β-D-glucanase, may be preferable to zymolyase because it may have greater activity against filamentous fungi. Following spheroplast induction, lysis of the cell membrane is accomplished with sodium dodecyl sulfate (SDS) and EDTA. Purification of released fungal DNA preparatory to PCR is accomplished with the QIAamp DNA Mini-Kit spin column (Qiagen, Inc., Valencia, CA, USA), which also includes a proteinase K digestion step. After adjusting the DNA concentration, PCR is accomplished using a kit, described below, for intrinsic labeling of the amplicon with digoxigenin. The post-PCR detection of the amplicon is also described below. When conducted by this method, the sensitivity for detecting *Candida* or *Aspergillus* spp. is reported to be 5 cfu ml^{-1} of blood.

Whole peripheral blood pretreated by the lysis-centrifugation kit

This uses the method described previously [14]. Instead of a 'home-brew' method for lysing leukocytes and erythrocytes, lysis-centrifugation blood culture tubes (Isolator™; Wampole Laboratories, Inc, Cranbury, NJ, USA) can accomplish this phase of the procedure. While adding to the cost, it introduces an additional standardization element in the specialty laboratory where, it is believed, these tests will be performed under conditions of maximum quality control. There are minor but important differences between the original report [14] and that of [9]. After recovery of the fungal elements by centrifugation, a hot dilute alkali step improves the detection of *Aspergillus* spp. The subsequent use of Qiagen spin columns (Qiagen, Inc.) in the DNA purification step avoids the need for phenol–chloroform extraction.

Sample preparation from blood culture broth

This uses the method described previously [15]. Simpler sample preparation is possible if whole peripheral blood is incubated for a brief period in blood culture broth bottles (i.e., BacT/Alert® blood culture bottles; Organon Teknika Corporation, Durham, NC, USA). This preliminary incubation dilutes inhibitors of PCR, which may be present, and allows some natural amplification of the cfu. The method was designed to amplify *Candida* spp. DNA and its suitability for opportunistic molds remains to be determined. Blood culture broth, 200 μl, is combined with Tris buffer-Triton X-100 detergent, boiled for 15 min and then shaken with zirconium beads prior to PCR. A preliminary incubation in the blood culture bottles for 3 h is sufficient to detect 5 cfu ml^{-1} of *Candida* spp. [16].

Total DNA sample preparation from whole blood

See section 'PCR assays for the diagnosis of invasive mycoses'.

Target selection for PCR amplification: rDNA sequences

Factors downstream from sample preparation, which promote a favorable outcome of clinical PCR, are: universal fungal primers, multicopy gene targets and species–specific probes. These requirements can be met by selecting, as the target for PCR, the ribosomal RNA (rRNA) genes of fungi, a tandem array of 50–100 copies in the haploid genome. Two parallel approaches have been taken in selecting targets. First, the 18S (small subunit [SSU]) rDNA [12]: a primer pair selected to bind at nucleotide positions 544–563 and 1033–1014 in the SSU rDNA amplifies a 482–503-bp fragment. Oligonucleotide probes bind in the V7 to V9 region of the SSU. Species probes for five *Candida* spp. and an *Aspergillus* genus probe were devised. Alternatively, a suitable target for PCR is the intergenic transcribed spacer region 2 (ITS2) lying between the 5·8S and 28S rRNA genes and amplified by universal fungal primers ITS3 and ITS4 [17]. The size of ITS2 for 16 *Candida* spp. ranges 66–240 bp not including the flanking regions of the 5·8S and 28S subunits. Although small in size, this target region has been fruitful in allowing the design of 16 *Candida* species-specific probes [17] and five *Aspergillus* species probes [18]. The sequences of many of these probes have been published [17] and for the remaining probes, information can be obtained from C.J. Morrison at the CDC (Atlanta, GA, USA): cjm3@cdc.gov. The ITS2 regions have been patented, but may be used for research purposes, and agreements to obtain the probes can be arranged.

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PCR and detection phase

A standard approach to detect PCR amplicons is the PCR-enzyme linked immunosorbent assay (ELISA). Biotinylated oligonucleotide probes may either be bound to streptavidin-coated wells of plastic microtiter plates, or may be added into the fluid phase. Probes are hybridized with the digoxigenin-labeled amplicon and the complex is trapped on the solid-phase plastic wells. The original prototype of this method was that of Fujita *et al*. [14] which, with minor variation, is available in two commercial kits: the PCR-Dig-labeling kit (Roche Diagnostics Corp., Indianapolis, IN, USA) and the PCR-ELISA Dig-detection (Roche) kit. Immunodetection of the digoxigenin-labeled amplicon bound to the solid phase is accomplished by a colorimetric immunoperoxidase reaction.

Aspergillus spp. detection from whole blood specimens

This uses the method described previously [9]. Whole blood specimens obtained 1–3 times week^{-1} were screened by PCR from 36 neutropenic patients receiving 400 mg fluconazole day^{-1} as suppressive therapy. Fourteen patients developed definite or probable IA; each patient had at least one positive PCR test result. Sixty-three of 142 (45%) PCR tests were positive in this group. As controls, healthy donors were unreactive, and PCR tests of blood from 11 neutropenic patients with no laboratory or clinical evidence of IA produced five positive PCR results out of 58 blood samples (8% positive). Subsequently, Loeffler [19] observed that it was possible to detect extracellular DNA in plasma or serum but that only 54% of whole blood specimens that were PCR-positive were also positive when plasma was the starting material for PCR.

Galactomannan detection in comparison with PCR detection in serum of patients with IA

This uses the method described previously [20]. The detection of serum galactomannan (GM) in a double-antibody sandwich enzyme immunoassay (EIA) with a monoclonal antibody directed against a galactofuranose epitope is the basis of the *Aspergillus* Plateleia EIA marketed in Europe by Sanofi Diagnostics Pasteur (Chaska, MN, USA), now acquired by Bio-Rad Inc. (Hercules, CA, USA). A retrospective study of 281 serum samples collected weekly from 41 neutropenic and/or systemic steroid treated hematology/oncology patients was conducted to evaluate the utility of serum GM in comparison with serum-PCR. For the PCR assay, 200 µl of serum, processed by QIAamp spin-column kit (Qiagen, Inc.) was directly applied in PCR, with fungal mitochondrial DNA (mtDNA) as the target sequence. Amplicons were detected in a microtitration EIA utilizing biotin-probes specific for *Aspergillus*. Of 13 patients who were both PCR-positive and GM-positive, all had IA. Of six patients whose serum tested positive for GM but were negative in PCR, five had IA. Four of 23 patients who were negative in both tests had IA. In summary, no patients had a positive serum-PCR and a negative serum-GM test. Consequently, the savings in sample preparation by direct PCR of serum did not appear to improve diagnostic sensitivity. Perhaps whole blood specimens would result in a greater yield of positive PCR results.

As experience is gained with the monoclonal sandwich EIA for serum-GM, a tendency for false-positive tests has been detected [21] and related to a variety of conditions, i.e., subclinical infection, intestinal colonization, cross-reactions with cyclophosphamide, adsorption of dietary GM from a damaged gut. False-positive results can be reduced if two consecutive positive serum specimens are required to indicate a positive test. False-negative results can also occur.

D-Arabinitol (DA) as a marker for IC

DA, a five carbon polyol, is a marker of IC resulting from infection with *C. albicans* and other *Candida* spp. [22]. DA is produced by the major clinically important *Candida* spp. except for *C. krusei*, and its production by *C. glabrata* is uncertain. Technology for DA detection has evolved significantly from early prototype gas-liquid chromatography (GC) reviewed in [23]. DA is a *Candida* spp. metabolite and it is questionable as to whether it is produced at all by humans. Whether it is natural or present as a result of dietary or microbial DA absorbed by the gut, it is necessary to normalize the concentration of DA by accounting for possible renal impairment. This is accomplished by reporting serum DA as the DA/creatinine (Cr) ratio. L-Arabinitol (LA) appears to be a natural metabolite of humans, so that a DA/LA ratio is an alternative means of normalizing the amount of microbial DA. DA in serum or urine is a diagnostic marker of IC and can be measured by enzymatic-fluorometric, enzymatic-colorimetric methods, or by GC-mass spectrometry (GC-MS).

Enzymatic method for D-arabinitol

The enzymatic method for detecting DA requires DA dehydrogenase as a consumable reagent. The reaction catalyzed by DA dehydrogenase is an NAD linked oxida-

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tion of DA to D-ribulose. The concomitant production of NADH can be detected spectrofluorophotometrically in a tetrazolium dye reduction reaction [24]. The test is available in kit form from Nacalai Tesgue (Kyoto, Japan). Recently, another variation was reported utilizing recombinant D-arabinitol dehydrogenase cloned from *C. albicans* [25], but that enzyme is not available commercially. The initial rate of NADH production is measured by spectrofluorophotometry in a Cobas Fara II centrifugal analyzer (Roche), and in the same run creatinine can also be measured. The DA/Cr units, expressed as $\mu M\ mg^{-1}\ dl^{-1}$, were reported as $1{\cdot}1\ \mu M\ mg^{-1}\ dl^{-1}$ for healthy controls, and in 11 candidemia patients as mean $2{\cdot}74\ \mu M\ mg^{-1}\ dl^{-1}$, range $1{\cdot}6$–$19{\cdot}1\ \mu M\ mg^{-1}\ dl^{-1}$. An earlier version of the enzymatic method for DA was evaluated in a multicenter study by Walsh *et al*. [26]. They found that the DA/Cr ratio was significantly elevated in 25 of 30 (83%) patients with persistent fungemia. Moreover, elevated DA/Cr preceded positive blood cultures up to 50% of the time. The test was prognostic and fluctuations could be used to monitor the therapeutic response. About the same time, Tokunaga *et al*. [24] evaluated the enzymatic-fluorometric kit method in patients with disseminated candidiasis, pyelonephritis, or urinary tract colonization. Their units differed from those used by Walsh *et al*. [26], but they found elevated DA/Cr in nine of 11 (81%) patients with disseminated candidiasis. Only two of 16 (12·5%) patients with urinary tract colonization had elevated DA/Cr.

DA/LA determination by GC-MS

Although clinical microbiologists might find GC-MS an unfamiliar technology, bench top units with chemical ionization detectors are becoming more common in major medical centers of developed countries. The availability of chiral columns facilitates separation and quantitation of the two enantiomers, DA and LA. Patient urine was determined to be a suitable specimen for analysis and significantly elevated urine-DA/LA ratios were found in 15 of 17 (88%) acute leukemia patients with IC [27] and in all 10 pediatric cancer patients with confirmed IC who were tested [28].

PCR assays for the diagnosis of invasive mycoses

PCR has advantages over other diagnostic methods, in that very low target numbers of pathogenic fungal DNA may be amplified and detected without the need for viable fungal cells to be present. PCR is relatively rapid, often giving a result 4–8 h after sample collection. With these factors in mind, a feasibility study was conducted on the capacity of PCR assays to detect *Aspergillus* and/or *Candida* species in clinical specimens. The goals for a clinically useful *Aspergillus* PCR are to: (i) develop a sensitive and specific assay to detect *Aspergillus* DNA in blood and bronchoalveolar lavage (BAL) fluid early in the disease process; (ii) develop a rapid, standard method to isolate and concentrate DNA from clinical specimens; (iii) determine the clinical relevance of PCR test results.

Following the format of other clinical PCR assays developed by Roche Diagnostics Systems, the *Aspergillus* assay uses microtiter plates and a colorimetric immunoenzymatic detection format (PCR-ELISA; Roche). A low copy number internal control plasmid is added to the PCR master mix to monitor inhibition, and deoxy uracil triphosphate (dUTP) and Amperase® (Roche) are incorporated to prevent carry-over contamination. The internal control DNA shares the same primer-binding site as the target, but has a different probe-binding region. The gene target for *Aspergillus* species PCR is a 500-bp fragment of the 18S multi-copy rRNA gene. Although the primers are specific for both *Aspergillus* and *Penicillium* species, a probe (Roche) was designed to discriminate between the two genera. *P. marneffei*, however, a pathogen found in southeast Asia is detected by the *Aspergillus* probe.

This *Aspergillus* PCR assay was previously shown to be both sensitive and specific when BAL specimens were used [29]. Of 86 specimens analyzed, cultures and PCR tests detected evidence of *Aspergillus* species in 21 (24·4%). One BAL specimen was culture-positive but PCR-negative; but PCR detected 13 additional *Aspergillus*-positive specimens, which were culture-negative. To be of most benefit, an assay should be effective before, or as soon as clinical symptoms appear, and on specimens which are not invasively procured. Accordingly, whole blood was selected as the specimen of choice.

Total sample preparation from whole blood

A total DNA capture method utilizes buffers commercially available from Roche Molecular Biochemicals (Indianapolis, IN, USA), Roche Diagnostics Systems (Indianapolis, IN, USA) and Qiagen DNA midi-prep columns (Qiagen, Inc). Many published *Aspergillus* blood extraction protocols exist, but the present method is the only one, to our knowledge, that does not require fractionation of whole blood components prior to nucleic acid isolation. Both *Aspergillus* spp. conidia and extracellular or 'free' DNA can be captured and concentrated with this method. Other methods, in contrast, rely on erythrocyte and leukocyte lysis steps and the subsequent

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centrifugation of hyphal elements, yeast cells (in the case of *Candida* other yeast), or conidia. This type of fractionation complicates detection of circulating free DNA.

Sample preparation begins with 1 ml of whole blood, which is incubated with 1 ml of Amplicor HIV Extraction Reagent (Roche Diagnostics Systems), and 200 μl of Proteinase K solution at 70 °C for 30 min on a shaker platform. A 6-ml aliquot of RNA/DNA Stabilization Reagent for Blood/Bone Marrow (Roche Molecular Biochemicals) is then added, and the mixture is incubated at 95 °C for 30 min. A 4-ml aliquot of absolute ethanol is then added and the lysate is loaded and centrifuged through a Qiagen column from the QIAamp DNA Blood Midi Kit (Qiagen, Inc). Protocols and buffers are used to complete purification of the DNA, according to the manufacturer's instructions. This protocol has also been successfully used on serum.

To assess the recovery of DNA using this method, a positive control plasmid DNA of known copy number was artificially added to serum or whole blood, processed, and PCR-amplified for 45 cycles. Recovery of DNA was determined by a quantitative PCR assay similar to that used in Amplicor Monitor® (Roche) assays. [30]. The results indicated recovery rates of 33–100%, with a trend toward higher recoveries at higher target inputs. A dilution series of *Aspergillus* sp. conidia artificially inoculated into 1 ml of blood was processed and analyzed to assess fungal cell lysis and recovery. *Aspergillus* sp. DNA could be detected from as few as one conidium which contains ~100 copies of the 18S rRNA gene. Comparison of this sample preparation protocol to a published protocol [9] that incorporates erythrocyte and leukocyte lysis and separation prior to using Qiagen technology, shows that methods to lyse the formed elements of the blood resulted in losses in recovery of both artificially added *Aspergillus* sp. genomic DNA and DNA released from *Aspergillus* sp. conidia.

A universal sample preparation method facilitated analysis of both the centrifuged leukocyte fraction and the supernatant from lysis of erythrocytes from a specimen of whole blood obtained from an IA patient. Quadruplicate extractions were performed, and all four supernatants were PCR-positive for *Aspergillus* sp. DNA, whereas only two of the four leukocyte fractions were PCR positive. These results underline the possibility that the leukocyte-containing fraction may not provide the greatest sensitivity to detect *Aspergillus* sp. DNA. Experience with whole blood specimens from patients with confirmed IA, however limited, has shown that *Aspergillus* DNA can indeed be recovered from whole blood, even before BAL procedures are performed, whereas in some blood specimens PCR is not positive for *Aspergillus* until clinical suspicion is raised to the point that a BAL procedure is performed. The reproducible correlation of PCR with confirmed IA remains to be established.

Cross-contamination from environmental sources is a potential pitfall hindering the feasibility of *Aspergillus* PCR assays. When such contamination occurs, false positive results may be obtained, or false negative results may arise if environmental contaminating DNA outcompetes low-level, true positive specimens. For example, analysis of approximately 80 peripheral blood specimens from a clinical population of 29 patients with no suspected IA revealed that 26 patients had at least one *Aspergillus*-positive PCR result. Negative control blood specimens from other donors and reagent controls, however, were negative, suggesting that the specimens became contaminated during routine collection or in the dispensing of aliquots. Another potential source of environmental contamination is the storage location of PCR reagents. A lot of *Aspergillus* master mix failed with respect to sensitivity because a *Penicillium* species had grown on a cardboard box in the 4 °C refrigerator, contaminating the master mix with such high copies of *Penicillium* DNA that low-level amplification of *Aspergillus* sp. DNA was out-competed. Consequently, special care must be taken in the manufacture of reagents, and in ensuring that the environment in which the samples and reagents are stored is as free as possible from fungal contamination.

Reverse line-blot assay to detect *Candida* species DNA

This *Candida* PCR assay, also based on SSU rRNA gene sequences, was designed to amplify and detect all *Candida* species, and incorporates at least 10 probes to differentiate as many individual *Candida* species. Although *C. albicans* is responsible for the majority of incidents of candidemia, in recent years other *Candida* species have emerged as a result of the increasing use of prophylactic antifungal therapy. *C. glabrata* and *C. krusei* are notable because of innate decreased susceptibility to fluconazole. There is a larger population of immunosuppressed patients or those who, while not deliberately immunosuppressed, are vulnerable to candidemia and IC because of prolonged hospital stays, cardiothoracic or abdominal surgery, in conjunction with the use of intravascular devices and broad spectrum antibacterial therapy. In these patients rapid therapeutic intervention is necessary to control IC. Emphasis has shifted to the development of PCR-based diagnosis from blood specimens because of the relative insensitivity of culture methods, and because alternative non-culture methods remain promising proto-

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types and have not reached the point of clinical utility or commercial availability. The goals for a *Candida* species PCR assay are to: (i) develop a sensitive and *Candida* genus-specific amplification and genus-and species-specific detection; (ii) switch from microwell plate detection to a 'reverse-line-blot' format that will allow 30 or more probes to be covalently linked to a nylon membrane for simultaneous detection and species identification; and (iii) determine the clinical relevance of the reverse-line-blot PCR assay to detect candidemia from whole blood specimens.

The nylon membrane strip is charged with the following probes: *Candida*-genus, *C. albicans*, *C. dubliniensis*, *C. tropicalis*, *C. viswanathii*, *C. glabrata*, *C. kefyr*, *C. lusitaniae*, *C. guilliermondii*, *C. krusei* and a probe to distinguish *S. cerevisiae*, which is also amplified by the primer pair. A diluted genus probe is used to ensure detection of low-copy number signals. An additional specific probe is added which is designed to hybridize to an internal control plasmid to monitor any inhibitors of PCR amplification that may be present in the clinical blood specimen. After using the total DNA sample preparation from whole blood (see above) inclusivity and exclusivity panels were performed in the reverse-line-blot assay and the results showed that all *Candida* species tested were detected and amplified, except for *C. rugosa*, the detection of which can be accomplished with an additional primer pair.

The combination of the total DNA sample preparation from whole blood and the reverse line-blot PCR assay enabled the detection of one *C. albicans* cell artificially added to 1 ml of whole blood and 10–100 plasmid gene copies. The *Candida* reverse line-blot PCR assay was evaluated on a limited number of clinical specimens. The evaluation of the reverse line-blot PCR assay in peripheral blood specimens from patients is being expanded to establish the test's clinical utility.

Plasma (1,3)-β-D-glucan determination in patients with invasive fungal infections

Invasive deep mycoses and fungal sepsis are frequent infectious complications in patients with hematologic malignancies [31]. Early recognition of the invading fungi is important for their successful treatment. Blood culture is the gold standard for fungal sepsis; however, it is not always useful for early diagnosis and its sensitivity is not satisfactory [7]. Hence, recent efforts to improve the efficiency of diagnostic tests for invasive fungal infections have been directed toward the detection of fungal components or metabolites. (1,3)-β-D-Glucan is a characteristic cell wall component of fungi with the exception of the zygomycetes. Other microbes, as well as the cells and extracellular fluids of animals, all lack this polysaccharide. This polysaccharide should therefore be a good indicator of systemic fungal infection if detected in blood or in other normally sterile body fluids. Recently, we developed a method to detect (1,3)-β-D-glucan with high sensitivity and specificity and its usefulness for the diagnosis of invasive fungal infections is described herein.

Method for determination of plasma (1,3)-β-D-glucan

Before the advent of a specific (1,3)-β-D-glucan assay, its plasma concentration was estimated from the difference between measurements by a conventional chromogenic *Limulus* amoebacyte lysate test (Toxicolor™; Seikagaku Corporation, Tokyo, Japan) [32] and an endotoxin-specific *Limulus* test (Endospecy™; Seikagaku Corporation) [33]. Thus, the difference between these tests' values reflects (1,3)-β-D-glucan concentration, and was designated as the Fungal Index [34]. The coefficient of variation of either test was $\leq 4\%$, and the detection limit 1 pg ml^{-1} [33]. The reference value for normal individuals was less than 10 pg ml^{-1} [35].

The direct measurement of plasma (1,3)-β-D-glucan was then developed using factor G, a horseshoe crab coagulation factor extremely sensitive to this polysaccharide (Fungitec G test; Seikagaku Corporation) [36]. In preliminary experiments, this test was used to detect (1,3)-β-D-glucan contained in yeast or mycelia of various fungi grown *in vitro*, such as *C. albicans*, *Microsporum canis*, *Trichophyton mentagrophytes*, *T. rubrum*, *Saccharomyces cerevisiae*, *A. flavus*, *A. fumigatus* and *Cryptococcus neoformans*. The test reacted in a dose-dependent manner with each fungal extract [37]. Plasma (1,3)-β-D-glucan levels in 79 plasma samples obtained from febrile neutropenic patients by both the conventional subtraction method and the new assay were determined. The correlation of the Fungal Index and the G-test was high ($r = 0{\cdot}90$).

Plasma (1,3)-β-D-glucan determination in patients with invasive fungal infections

To examine the clinical utility of (1,3)-β-D-glucan determination, plasma levels in patients with deep-seated mycoses were analyzed at Jichi Medical School Hospital. Forty-three episodes of fungemia were selected for study from hematology-oncology patients treated during the period 1978–1991. The distribution of isolated fungi was 39 yeast cultures (*Candida* spp., also including one *Saccharomyces* isolate and one *Trichosporon* sp.) and one each

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of *Acremonium* sp. and *Fusarium* sp. Before 1986, 14 patients were diagnosed by blood culture and treated with amphotericin B. Only two received antifungal therapy within 3 days of onset. In 1986, the glucan assay by Fungal Index was introduced. Concentrations of plasma (1,3)-β-D-glucan were ≥ 10 pg ml^{-1} in 27 of 29 patients tested. Furthermore, 16 of the 29 patients received antifungal therapy within 3 days or even before the documentation of fungemia because of the elevation of plasma (1,3)-β-D-glucan. Survival was significantly improved by the introduction of this early treatment. Because most of the deaths occurred within 2 weeks after documentation of fungemia, we classified death as fungemic when it occurred within that period. Overall mortality from fungemia was 62·8% (27 of 43). Before the introduction of the Fungal Index, fungemic mortality was 85·7% (12 of 14); it was reduced to 51·7% (15 of 29) thereafter ($P = 0{\cdot}01$) [38]. Although the test is unable to determine the species of fungi, it is helpful to know as early as possible whether the patient is suffering from a deep-seated mycosis or not and to initiate antifungal therapy without delay. Results of the plasma (1,3)-β-D-glucan test can be available within 2 h, making it a useful screening test for invasive fungal infections and fungemia including those caused by uncommon fungi such as species of *Fusarium*, *Trichosporon*, *Acremonium* and *S. cerevisiae* [35].

The clinical usefulness of a method for the direct measurement of plasma (1,3)-β-D-glucan (Fungitec G test) [36] for diagnosing invasive fungal infections was examined in a multicenter trial in Japan [39]. Of 202 febrile episodes, 41 were due to infections caused by species of *Candida*, *Aspergillus*, *Cryptococcus* and *Trichosporon*. Fifty-nine episodes resulted from infection of non-fungal etiology, namely Gram-negative or Gram-positive bacterial infections or febrile responses to drug therapy, and 102 were of unknown origin. Although the normal concentration of plasma (1,3)-β-D-glucan never exceeded 10 pg ml^{-1}, fungal febrile episodes could be almost completely separated from non-fungal episodes by a line drawn at 20 pg ml^{-1}. With that concentration as a cut-off, 37 of the 41 fungal infections were positive (sensitivity 90%) and all 59 non-fungal infections were negative (specificity 100%). This high specificity will help prevent the indiscriminate use of antifungal agents and avoid the development of resistant strains.

Plasma (1,3)-β-D-glucan determination in patients with febrile neutropenia

In a multicenter study [39], 102 patients were diagnosed with fever of unknown origin. Among these patients, plasma (1,3)-β-D-glucan concentrations were elevated in 26 (25·5%). These patients with positive glucan tests might have been suffering from an occult deep mycosis. We analyzed the response to antifungal therapy in 45 of these patients with neutropenia and fever unresponsive to antibiotics. The end-point was that fever resolved after 2 weeks of antifungal therapy. Plasma β-glucan concentrations assessed by the Fungal Index were elevated (≥ 10 pg ml^{-1}) in 22 patients and were below that concentration in 23 patients at the time of antifungal therapy. The efficacy of intravenous antifungal therapy with azoles (miconazole or fluconazole) was significantly higher in the high plasma (1,3)-β-D-glucan group (18 of 22 patients, 81·8%) than in the low plasma (1,3)-β-D-glucan group (10 of 23 patients, 43·5%). Plasma (1,3)-β-D-glucan concentrations were also determined after antifungal therapy in 16 patients of the high plasma (1,3)-β-D-glucan group. It decreased significantly in all the responders ($n = 12$), whereas it was stable ($n = 2$) or elevated ($n = 2$) in non-responders. In the low plasma (1,3)-β-D-glucan group, the change of plasma concentrations was unremarkable in 16 (five responders and 11 non-responders), and was elevated in two non-responders. These results indicate that β-glucan detection is useful to differentiate fungal infection from non-fungal infection in patients with persistent fever of unknown origin.

Recently, the recommended guidelines for the use of antibiotics and antifungal agents were published by the Infectious Diseases Society of America [40]. Addition of amphotericin B is recommended at day 5 if fever has continued and persistent neutropenia is likely. Although the incidence of invasive fungal infections increases in patients with persistent fever, considerable numbers of patients will be suffering from infections of non-fungal etiology, such as those caused by antibiotic-resistant bacteria or by viruses. The determination of (1,3)-β-D-glucan seems useful to decide when to initiate antifungal therapy in patients with persistent fever. The validity of this approach should be confirmed in well-designed clinical trials, and such efforts will add to the scientific basis of empiric antifungal therapy.

Immuno- and molecular diagnosis of histoplasmosis

Histoplasmosis, a systemic fungal disease caused by *Histoplasma capsulatum* var. *capsulatum* [41], is an important health problem world-wide. Although the majority of cases present as mild-to-moderate flu-like disease requiring only supportive therapy, approximately 5% of patients experience more serious pulmonary and extrapulmonary disease which can be life-threatening if diag-

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nosis is delayed or if treatment is not initiated rapidly. Histoplasmosis is world-wide in distribution, with endemic areas containing the highest known concentration of cases located in the USA [42]. Smaller endemic regions are scattered in most of Latin America [43–45]. Histoplasmosis is one of the most common systemic mycoses in Brazil where epidemiological surveys carried out using the histoplasmin skin tests indicated that this mycosis is endemic in all areas surveyed [46]. In Rio de Janeiro State, over a 2-year period (1981–1982), follow-up of 43 cases of histoplasmosis was undertaken. This cohort consisted of 40 cases of acute pulmonary histoplasmosis (four outbreaks involving 35 individuals and five isolated cases), two patients with chronic pulmonary disease, and one patient with the chronic disseminated histoplasmosis [47]. Cases of histoplasmosis have been reported from states as far apart as Rio Grande del Sul and Amazonas. In the latter state, a 1993 outbreak occurred in seven Japanese tourists visiting a cave in Manaus [48]. These data suggest that cases of histoplasmosis in Brazil may be underestimated, and that a broader range of endemic areas occurs than was previously thought to be the case. The diagnosis of histoplasmosis is usually made by a combination of culture, histopathology, and measurement of antibodies or detection of antigen. However, each of these approaches has limitations. The isolation of the fungus is time-consuming and *H. capsulatum* often fails to grow from clinical specimens [41,49,50]. Serological evidence is the prime diagnostic indicator of histoplasmosis, but when conventional serology, such as complement fixation (CF) tests are used, a tendency exists for cross reactions to occur with sera from patients with blastomycosis, coccidioidomycosis and paracoccidioidomycosis. Immunodiffusion (ID) tests, while highly specific, may not convert to positive until 4–6 weeks after symptoms first appear. Serological responses in immunosuppressed patients are less reliable [1].

The antigenic complex most widely used for probing the immune response in histoplasmosis is histoplasmin [1], an unpurified culture supernatant from mycelial form cultures of *H. capsulatum* grown on synthetic medium. Histoplasmin contains several antigens, including the common C antigen (a heat-stable cross-reactive GM shared by the major genera of systemic dimorphic fungal pathogens) and the species-specific H and M glycoproteins [51]. Histoplasmin also contains a 62 kDa heat-shock protein that has been shown to mediate protection, but curiously this antigen does not elicit precipitins [52]. H and M antigens are potent stimulants of humoral and T-cell-mediated immunity. Precipitins against M antigen are the first to appear and form the basis of a specific immunodiagnosis. H and M antigens are considered the immunodominant antigens of *H. capsulatum* because they are expressed during infection and evoke complement-fixing and precipitins. These antigens are markers of disease activity in histoplasmosis patients whose immune response is intact. Moreover, H and M antigens are not found in morphologically similar non-pathogenic molds, which produce tuberculate macroconidia (*Chrysosporium* spp., *Sepedonium* spp.). For the latter reason H and M antigens are used in the exoantigen test for immunoidentification of *H. capsulatum* cultures.

To develop new diagnostic tests for histoplasmosis, we purified and monitored the physical, chemical and serological properties of the H and M antigen. Tandem cation exchange chromatography using preliminary CM Sepharose CL-6B followed by fast protein liquid chromatography on a strong cation exchanger is an appropriate method to purify H, but especially suitable for the immunodominant M antigen [53]. Both H and M antigens are glycoproteins with relative masses of 120 and 94 kDa, respectively, and they contain species-specific protein epitopes and glycosidic epitopes which are *N*-linked to the peptide core and probably belong to the high mannose type glycan. Treatment of these proteins with *N*-glycosidase F produced pronounced shifts in their relative masses implying that a major portion of the carbohydrate epitopes are attached by *N*-glycosidic linkages to the peptide core. H and M glycoproteins are almost completely deglycosylated by mild sodium metaperiodate oxidation since, after treatment, both bound only weakly to the lectin ConA. The glycosidic determinants that are naturally coupled to both antigens share epitopes with the polysaccharide C antigen, and are responsible for the cross-reactivity observed in immunoblots [54]. Consequently, the presence of these moieties is an obstacle to diagnostic specificity and deglycosylation of M glycoprotein increases its specificity for the diagnosis of histoplasmosis in primary binding immunoassays.

We evaluated a WB test as a suitable method to detect antibodies against M antigen by comparing its glycosylated and deglycosylated forms. Sera from patients with histoplasmosis, paracoccidioidomycosis, blastomycosis, coccidioidomycosis, aspergillosis and tuberculosis were tested by WB against glycosylated M-antigen. This assay showed 100% sensitivity when histoplasmosis serum samples were tested, with reactions directed against the 94 kDa glycoprotein M antigen. Although the WB is a highly sensitive test, it is not specific for histoplasmosis when glycosylated M-protein is used as antigen: a total of 81% of paracoccidioidomycosis, 25% of blastomycosis, 33% of coccidioidomycosis, 73% of aspergillosis and 16% of tuberculosis serum samples cross-reacted with M anti-

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gen and with large amounts of the polydisperse C antigen. When serum samples were analyzed by WB to compare untreated M glycoprotein with the M antigen altered by periodate oxidation or by deglycosylation with endoglycosidases, cross-reactions with heterologous sera could be attributed to periodate-sensitive carbohydrate epitopes, as reflected by the increase of 46·1% to 91·2% in test specificity after periodate treatment [55].

A WB assay for antibodies against M antigen is a potential diagnostic test for histoplasmosis provided that periodate-treated M-protein is used as antigen. The WB test was evaluated to detect antibodies against native glycosylated and chemically deglycosylated M and H antigens of *H. capsulatum* in serum obtained from patients during the acute phase of an outbreak of pulmonary histoplasmosis at a USA correctional institution [56]. Of 275 serum samples tested by ID and CF tests, serum samples from 49 patients from this outbreak and those from 37 negative controls were tested by WB. Serum specimens from a group of patients were tested including from patients whose sera were negative for CF-antibodies and precipitins early in the acute stage of histoplasmosis, but who sero-converted during convalescence 4-weeks later. Antibodies against untreated H and M antigens were detected at a 1:100 dilution by WB in nine out of 20 (45%) of the acute phase serum samples and in all 20 convalescent phase specimens. The WB test's sensitivity for acute phase specimens increased to 18/20 (90%) when H and M antigens were treated by periodate oxidation to inactivate susceptible carbohydrate epitopes. When native glycosylated antigens were used in WB, positive reactions were observed in negative control serum specimens (3/37; 8·1%), and in those obtained from asymptomatic persons from the outbreak investigation (13/20; 65%). These false-positive reactions were also attributed to glycosidic epitopes since the specificity of the WB test increased from 63·6 to 98·5% when periodate-treated H and M antigens were used. WB with deglycosylated H and M antigens of histoplasmin provides a rapid, sensitive and specific test to diagnose acute pulmonary histoplasmosis before precipitins can be detected. Chemical deglycosylation of this antigen allowed the detection of antibodies earlier in the acute stage of pulmonary histoplasmosis than had been previously possible.

At present, the WB test is being evaluated as a confirmatory diagnostic method for histoplasmosis. Among a group of seven patients with strong evidence of histoplasmosis whose sera were negative for precipitins, antibodies against H and M antigens were detected in six patients by WB probed with purified H and M antigens, and with recombinant M antigen. The WB test is useful to identify cases early in infection, before seroconversion can be detected by ID.

'Dip-stick' test

This test was developed to simplify the detection of anti-*H. capsulatum* antibodies by using a format which could be easily applied in other laboratories without the need for special apparatus, and is suitable for conducting sero-epidemiologic surveys. Serum samples from 75 individuals were analyzed by a 'dip-stick test', i.e., a dot immunobinding assay on nylon membrane strips to which purified fraction P-II was affixed. This fraction contains mainly M antigen [57]. Thirty serum samples were obtained from patients with culture-confirmed histoplasmosis and 45 remaining samples were obtained from patients with other mycoses, with tuberculosis, and from healthy persons. In this dip-stick assay, 27 of the 30 (90%) serum samples from histoplasmosis patients reacted positively, giving results similar those observed in precipitin tests. Cross-reactions were observed in 12 serum samples obtained from patients with other mycoses. Although, in its present form, the test's specificity is only 71% the dip-stick test is promising as a rapid presumptive screening method for histoplasmosis. The test specificity will probably improve if the partially purified histoplasmin is treated by mild periodate oxidation.

Despite the accumulated evidence of its importance in humoral immune responses, as measured in clinical serological tests, the biological function of M antigen remained obscure, and its interaction with cells of the immune system was largely uncharacterized. The lack of availability of monomolecular M antigen has delayed efforts to determine whether it can induce protective immunity. Recombinant DNA technology was used to begin to address these aspects by cloning, expressing, and characterizing the gene encoding M protein [58]. Amino acid sequences from the N-terminus and from internal peptides were determined, and a significant sequence similarity was shared by two internal peptides of M protein with sequences of catalases of other fungi. This sequence homology facilitated the design of degenerate primers to isolate a gene fragment by PCR, which then was used to screen an *H. capsulatum* genomic library leading to the isolation, cloning, and sequencing of the full-length gene. The M gene consists of 2187 bp of DNA encoding a protein of 80719 Da, which has significant homology to catalases from *A. fumigatus*, *A. niger* and *Emericella nidulans*. A cDNA was generated by reverse transcription PCR and cloned into the expression vector pQE40. The identity of the cloned, expressed protein was confirmed

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by WB. The recombinant fusion protein was immunoreactive with monoclonal antibodies raised against M antigen; with monospecific polyclonal mouse anti-M antiserum, and with a serum sample from a histoplasmosis patient. The gene encoding the major immunodominant M antigen of *H. capsulatum* is a presumptive catalase and the recombinant protein retains serodiagnostic activity. The cloning and sequencing of the M gene and its expression as a recombinant protein open the possibility of mapping individual peptide epitopes to permit an analysis of B and T cell reactive sites within the protein. Additional knowledge gained in this respect would enhance understanding of the fungus–host interaction and would permit an exploration of the role of M antigen in the protective immune response. Moreover, these data may contribute to the development of antifungal therapy directed against fungal catalases. Recombinant M-antigen and/or selected peptide-epitopes derived therefrom will be useful to develop more sensitive and specific diagnostic tests. Studies are in progress in our laboratory to explore this last possibility.

Rapid identification of *H. capsulatum* cultures

The conventional exoantigen method, using the ID test, is a valuable tool for immunoidentification of mycelial forms of typical and atypical *H. capsulatum* cultures, although it can be time-consuming and false negative results can occur. Considering that immunoenzyme assays are more sensitive than precipitin methods, and that nucleic acid-based methods show higher specificity than immunological tests, alternative methods for rapid identification of *H. capsulatum* cultures have been developed and are being evaluated.

Dot-blot and WB assays

These two assays were compared with the traditional exoantigen test to detect soluble species-specific macromolecules produced by *H. capsulatum* cultures [59]. Duplicates of 5–7-day-old cultures grown in brain–heart infusion broth were used as source of exoantigens. After 24 h of Thimerosal sterilization, the exoantigens were obtained in the supernatant by concentration, and probed by dot-blot and WB against homologous rabbit anti-*H. capsulatum* serum, as well as being tested by ID. All the isolates studied were identified as *H. capsulatum* by these alternative methods. The dot-blot and WB tests showed higher sensitivity (12/12, 100%) than ID (4/12, 33%).

PCR for rapid identification of H. capsulatum *cultures*

A PCR assay for rapid identification of *H. capsulatum* cultures was accomplished using two oligonucleotide primers pairs, H1726F-H1972R and M4F-M8R, based on the sequences of the H and M genes, respectively [58,60,61]. With this PCR assay, 246 and 321 bp products were amplified successfully from 20 *H. capsulatum* strains, and the method showed 100% sensitivity. Studies are in progress to analyze the specificity of this PCR assay.

These two alternative methods to identify this pathogenic mould offer the potential to improve identification particularly when applied to atypical isolates.

Summary of non-culture methods

Looking ahead, how might these non-culture methods be implemented in the near future? First, we should acknowledge that non-culture methods are meant to supplement culture methods, not replace them. Rapid presumptive tests which are not species or even fungus-specific will have a place in the rule-out algorithm. In that category, kits to detect (1,3)-β-D-glucan now in use in Japan have value and, pending their wider availability, are suitable to be performed in the hospital laboratory. PCR with universal fungal primers has the capability to act as an ‘all fungus’ test, but its real utility is in providing species-specific data. Until there is a better understanding of the quality control measures needed to exclude false positives due to potential environmental contamination, among other issues, clinical PCR tests for systemic opportunistic yeasts and molds are best performed in a specialty laboratory. Genus-specific tests, for example, serum GM by sandwich EIA are appropriate and, pending general availability, are suitable to be performed in the hospital laboratory. The determination of DA in serum or urine is a promising independent alternative marker of infection. It continues to be refined and evaluated in different laboratories world-wide. This test, for the foreseeable future, seems destined to be performed in a specialty laboratory. Improvements in antibody testing as a diagnostic adjunct in acute pulmonary histoplasmosis are linked to the wider availability of deglycosylated, recombinant M-antigen.

Contributors

The contributors to this symposium were: **E. Reiss,** *PCR for* Candida *species and* Aspergillus *species from peripheral blood specimens*; **K. Orle,** *PCR assays for the diagnosis of invasive mycoses*; **M. Yoshida & T. Obayashi,**

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Plasma (1,3)-β-D-glucan determination in patients with invasive fungal infections; **R. M. Zancopé-Oliveira,** *Immuno- and molecular diagnosis of histoplasmosis*. The co-convenors were **E. Reiss** and **T. Obayashi**.

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