

Molecular Detection, not Extended Culture Incubation, Contributes to Diagnosis of Fungal Infection

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Abstract

Background. Despite of low sensitivity, fungal culture remains one of the key methods for diagnosing and treatment of fungal infections as it identifies etiology at genus and species level and offers susceptibility testing. The Manual of Clinical Microbiology recommends that fungal cultures screening for all pathogens should routinely be held for 4 weeks to maximize the recovery of slow growing species. Information on the optimal fungal culture time in the era of expansion of immunocompromised populations is lacking. The goal is *to review our experience with fungal culture in order to determine the optimal culture incubation time; to review our experience of broad-range ITS PCR for diagnosis of culture negative fungal infections.*

Methods. *Fungal culture and ITS PCR results from January 1, 2013 to December 31, 2017 were reviewed.*

Results. Ninety six percent of positive cultures (4058) were detected in the first seven days of incubation. During the second week of incubation, 2.8% of positives (111) were detected from day 8 to 10, and 1.7% (71) were detected from day 11 to 14. Only 0.1% of positive culture were detected in the third week of incubation, and no positive culture was detected in the fourth week of incubation. No Clinical significance of fungal isolates recovered after 14 days. Clinical significant pathogens were detected in 0.2% culture negative samples.

Conclusion. Extending culture incubation beyond two weeks did not generate clinical relevant results. When culture failed to make laboratory diagnosis, ITS PCR produced clinical significant results.

Introduction

While the number of immunocompromised patients increases as a result of the widespread adoption of aggressive immunosuppressive therapy and the use of new immune modifying drugs, invasive fungal infections becomes an emerging problem (1) because of the high mortality rate associated with these infections (2). The 12-week mortality rate of patients with invasive fungal infections was reported as high as 46.7% for adult hematopoietic stem cell transplant recipients and 29.6% for solid organ transplantation patients (3). Prompt initiation of antifungal therapy is the most important intervention to decrease mortality in patients with invasive fungal infection (4).

Despite of low sensitivity, fungal culture remains one of the key methods for diagnosing and treatment of fungal infections as it identifies etiology at genus and species level and offers susceptibility testing. Recovery of fungal pathogens relies on a numbers of factors, including specimen quality, appropriate specimen collection and transport, and optimal incubation condition and time. The Manual of Clinical Microbiology recommends that fungal cultures screening for all pathogens should routinely be held for 4 weeks to maximize the recovery of slow growing species. A few studies investigated the fungal culture incubation time and generated mixed results. Morris et al. reviewed over 2,700 consecutive clinical cultures and determined that 98% of fungal isolates were detected by day 14 (5). Labarca et al. evaluated close to 4,000 positive fungal cultures and concluded that isolates recovered during the fourth week were rarely clinically significant (6). Bosshard's study suggested that two weeks incubation was sufficient for non-dermatophytes evaluation, whereas a 4-week incubation was necessary for recovery of dermatophytes (7). Hove and Woods

reviewed their experience in an area endemic for *Histoplasma capsulatum*. Their study showed that 18 of 62 *H. capsulatum* isolates were recovered in week 3 and 4 (8).

To overcome the poor sensitivity of fungal culture, additional diagnostic tests were developed. Testing for antigen and fungal biomarkers are often used in addition to culture to help establish diagnosis (4). PCR-based assays targeting various genetic regions have been developed to improve the diagnostic yield, but mostly restricted to *Aspergillus* and *Candida* species (9). In the situations when other opportunist pathogens can't be ruled out, molecular detection of a broad range of pathogens is desired. Broad-range internal transcribed spacer (ITS) rRNA gene PCR followed by sequencing holds promise for detection and identification of fungal pathogens in human samples (10). In a recent prospectively performed study, ITS PCR showed to be helpful for diagnosis of microscopy-negative fungal infections (11).

ITS PCR was implemented in 2013 in our lab. Our protocol requires infectious disease physicians specially request the test when a specimen from sterile source is fungal culture negative and the suspension for fungal infections is high. The purpose of this study was to review our experience with fungal culture in order to determine the optimal culture incubation time; to review our experience of ITS PCR for diagnosis of culture negative fungal infections.

Materials And Methods

Study design – We retrospectively reviewed the positive fungal cultures of clinical significant specimens recovered in the Clinical Microbiology Laboratory at Northwestern Memorial Hospital, a 900-teaching hospital in Chicago, were included between January 1, 2013 to December 31, 2017. Specimen types include abscess, body fluids, bronchoalveolar lavage, endotracheal aspirate, sputum, skin, tissues, and wound. Information collected include fungal smear result, culture starting date, growth detection date, and species identification. Time to culture positivity was counted in days from culture started to the time when fungal growth was first detected.

Fungal stain and culture – Microscopic analysis of fungal smear was performed on all specimens with wet mount in 20% KOH and with Calcofluor-white staining. Smear of sterile body fluids was prepared with cytospin centrifugation. Clinical specimens were cultured on Inhibitory mold agar plate and Brain and Heart Infusion agar plate. Plates were incubated at 30 °C in ambient air for four weeks. All plates were examined three times a week during the two weeks of incubation and then once a week afterwards. The time to detection of fungal growth was counted in days from culture started to the time when fungal growth was first detected.

ITS PCR - For culture negative patients with high clinical suspicion for fungal infections, infectious disease physicians have access to order broad-range ITS PCR on the same specimen used for culture. When ITS PCR became positive, identification was determined with sequencing.

Nucleic acid extraction from clinical specimen was performed with the QIAamp DNA Mini Kit (QIAGEN, Hombrechtikon, Switzerland) following the manufacturer's instruction. PCR amplification of the ITS region was performed with primers ITS1 5'- TCCGTA GGTGAACCTGCG G- 3' and ITS4 5'- TCCTCCGCTTATGATATG C - 3'

as described previously (12). PCR product was purified with QIAquick PCR purification kit (QIAGEN, Hombrechtikon, Switzerland) and sequenced using BigDye kit (Life Technologies, Zug, Switzerland) and ABI 3130 Genetic analyzer kit (Life Technologies, Zug, Switzerland). Identification was obtained by BALST search against Genbank.

Medical record review – Electronic medical records were reviewed to determine the clinical significance of the organisms recovered by culture after 14 days of incubation and the organisms detected by ITS PCR. A result was determined to be clinically significant when the treating physician’s diagnosis based on the test result was recorded in the patient chart and patient was treated for the identified organism.

Results

Time to growth detection – Forty two hundred and thirty four non-duplicated positive cultures were included in the study. Table 1 listed the fungal species recovered and their corresponding numbers. Cultured specimens included abscess, blood, eye (corneal scraping, vitreous fluid), skin, respiratory tract samples (sputum, endotracheal aspirate, BAL fluid), sterile body fluids (CSF, pericardial, peritoneal, and synovial fluids), and tissues. Twenty eight percent of samples (1219) had positive fungal smear. Nearly ninety six percent of positive cultures (4058) were detected in the first seven days of incubation. During the second week of incubation, 2.8% of positives (111) were detected from day 8 to 10, and 1.7% (71) were detected from day 11 to 14. Only 0.1% of positive culture were detected in the third week of incubation, and no positive culture was detected in the fourth week of incubation (Table 1).

Table 1
Time to detection of fungal growth.

Species	Number (% of total)	Smear positive (%)	Days to positive <= 7	Days to positive 8-10	Days to positive 11-14	Days to positive 15-21	Days to positive 22-28
<i>Candida</i> spp.	3493 (82.6)	1009 (28.9)	3468 (99.3)	23 (0.6)	2 (0.1)	0 (0)	0
<i>Aspergillus</i> spp.	244 (5.8)	94 (38.5)	231 (94.7)	13 (5.3)	0 (0)	0 (0)	0
<i>Penicillium</i> spp.	117 (2.8)	2 (1.7)	85 (72.6)	14 (12)	15 (12.8)	3 (2.6)	0
Dimorphic fungi ^a	99 (2.3)	37 (37.4)	40 (40.4)	22 (22.2)	37 (37.4)	0 (0)	0
Other yeast ^b	59 (1.4)	20 (33.9)	58 (98.3)	1 (1.7)	0 (0)	0 (0)	0
Dematiaceous fungi ^c	67 (1.6)	10 (14.9)	36 (53.7)	20 (29.9)	11 (16.4)	0 (0)	0
Hyaline Hyphomycetes ^d	44 (1.0)	7 (15.9)	38 (86.4)	2 (4.5)	1 (2.3)	3 (6.8)	0
<i>Scedosporium</i> spp.	36 (0.9)	16 (44.4)	28 (77.8)	7 (19.4)	1 (2.8)	0 (0)	0
Zygomycetes ^e	34 (0.8)	15 (44.1)	34 (100)	0 (0)	0 (0)	0 (0)	0
Dermatophytes ^f	22 (0.5)	3 (13.6)	10 (45.4)	8 (36.4)	4 (18.2)	0 (0)	0
<i>Cryptococcus neoformans</i>	16 (0.3)	6 (37.5)	15 (93.8)	1 (6.2)	0 (0)	0 (0)	0
Total	4234	1219 (28.8)	4058 (95.8)	111 (2.6)	71 (1.7)	6 (0.1)	0

^aDiamorphic fungi include *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Sporothrix schenckii*; ^bOther yeasts include *Blastoschizomyces* spp., *Rhodotorula* spp., *Saccharomyces cerevisiae*, *Trichosporon* spp.; ^cDematiaceous fungi include *Alternaria* sp., *Aureobasidium* sp., *Cladosporium* spp., *Curvularia* spp., *Epicoccum* sp., *Exophiala* spp., *fonsecaea* sp., *Ochroconis* sp., *Phialophora* spp.; ^dHyaline Hyphomycetes include *acremonium* spp., *Beuveria* sp., *fusarium* spp., *Paecilomyces* spp., *Scopulariopsis* spp., *Trichoderma* sp.; ^eZygomycetes include *Cunninghamella* spp., *Mucor* spp., *Rhizopus* spp., *Rhizomucor* spp., *Syncephalastrum* sp.; ^fDermatophytes include *Trichophyton* spp., *Microsporum* spp..

Time required for recovery of different species varied. Of 4058 (95.8%) cultures growing yeast including *Candida* spp., *Cryptococcus neoformans*, and other yeast species, almost all of them, except two were recorded positive in less than 10 days of incubation. Growth of *Candida* spp. was detected in two cultures on

day 11. Growth of *Aspergillus* spp. and *Zygomycetes* was not detected after day 10. Fungal species belong to dimorphic fungi, dematiaceous fungi, and Dermatophytes required longer incubation time with growth of 59.6% dimorphics, 46.3% dematiaceous fungi, and 52.2% Dermatophytes was detected in the second week of incubation. The time to detection of different fungi groups was listed in Table 1.

Clinical significance of fungal isolates recovered after 14 days - The patients with a positive culture detected after 14 days were further investigated to determine the clinical significance of the recovered organisms. Three patients (patient 1, 2, 5) had no sign of infection at the specimen collection site and did not receive treatment for the recovered organism. The symptoms of the other three patients (patient 3, 4, 7) were attributed to either bacterial or viral infections based on the clinical course and treatment response. The recovered fungal organisms had not clinical significance. The last patient in the group (patient 6) was evaluated for presenting a lung mass. Bronchial wash was collected. Patient was later diagnosed with squamous cell lung carcinoma. *Penicillium* spp. recovered from the bronchial wash had no clinical significance. The summary of patient history and clinical information was listed in Table 2.

Table 2
Patients with positive culture after 14 days of incubation

Patient	Specimen	Culture Identification	Time to detection of growth (days)	Patient History	Reason for Culture	Clinical Information
1	Skin Biopsy	<i>Paecilomyces</i> spp.	19	Renal transplantation	One month of scattered papular lesions on extremities	No finding to suggest for infection by surgical pathological examination.
2	BAL	<i>Acremonium</i> spp.	20	Lung transplant	Left ureteral stone	No findings to suggest for infection by chest X-ray.
3	Sputum	<i>Acremonium</i> spp.	22	HIV on HAART	Suspected for hospital acquired pneumonia	Patient responded well to vancomycin and piperacillin/tazobactam treatment.
4	Sputum	<i>Acremonium</i> spp.	25	Bioprosthetic aortic valve	Hemoptysis with abnormal chest X ray	Hemoptysis resolved and serial CXRs showed resolution of persistent RLL infiltrate after empiric antibiotic treatment.
5	Wound	<i>Penicilium</i> spp.	15	Colon carcinoma and asthma	Evaluation prior to planned ventral hernia repair surgery	No infection
6	Bronchial wash	<i>Penicilium</i> spp.	15	No significant past medical history	Lung mass	Squamous cell carcinoma
7	BAL	<i>Penicilium</i> spp.	19	No significant past medical history	Cough, fatigue & decreased appetite	Upper respiratory viral infection

Table 3
Identification by ITS sequencing.

Patient History	Clinical Indication for fungal culture	Specimen	Direct Microscopic Examination	Identification by ITS Sequencing
Acute myeloid leukemia (AML)	Disseminated fungal infection involving multiple organs	Apical mass, liver biopsy, pericardium	Positive	<i>Saccaromyces cerevisiae</i>
Recent history of pneumonia requiring hospitalization	Left cerebellar lesion on imaging	Brain tissue	Positive	<i>Blastomyces dermatitidis</i>
Heart transplantation	Cavitary pneumonia	BAL	Negative	<i>Rhizopus microsporus</i>
Renal transplantation	Multiloculated cystic lesion medial to transplanted kidney in left pelvis	Blood	Negative	<i>Histoplasma capsulatum</i>
Hydrocephalus with a ventriculoperitoneal shunt	Brain lesion	Brain tissue	Positive	<i>Rhodotorula</i> spp.
Renal transplantation	Cavitary lung lesions/pulmonary nodule	CSF	Negative	<i>Aspergillus</i>
AML	Pneumonia, sinusitis and periorbital cellulitis	Ethmoid	Negative	<i>Fusarium</i> spp.
Stage III Wilms tumor	Skin lesion	Scalp lesion	Positive	<i>Rhizopus</i> spp.
AML	Cutaneous fungal lesions	Skin biopsy	Positive	<i>Fusarium</i> spp.
Pregnancy	Severe amniotic fluid infection	Placenta tissue	Positive	<i>Candida glabrata</i>
AML	Neutropenic fevers and pulmonary nodules	Tissue	Negative	<i>Aspergillus</i> spp.
AML	Disseminated fungal infection with empyema, pericarditis	Pericardial abscess	Positive	<i>Candida tropicalis</i>
Renal transplantation	Pulmonary cavitary lesion	TISC-left upper lobe region	Negative	<i>Cunningmahella</i> spp.
No significant medical history	Liver abscess	Liver biopsy	Positive	<i>Candida lusitaniae</i>
Renal transplantation	Disseminated invasive Aspergillosis involving multiple organs	Brain tissue	Positive	<i>Aspergillus</i> spp.
Renal transplantation	Mass in the left sinus invading the orbit	Eye tissue	Negative	<i>Aspergillus</i> spp.

Broad –range ITS PCR for diagnosis of fungal infections – 523 samples were tested with ITS PCR. Clinical significant pathogens were detected in culture negative samples from 16 patients, including five patients with acute myeloid leukemia, six transplantation patients, two patients with no significant past medical history, one cancer patient, one pregnant patient, and one patient with ventriculoperitoneal shunt for hydrocephalus. Presence of morphologically consistent fungal organism was observed on fungal smear in nine (56%) samples. The identifications were evaluated by the clinical team and the patients received treatment for the identified organisms.

Discussion

Our experience with fungal culture and ITS PCR from 2013 to 2017 was reviewed in this study. Forty two hundred and thirty four non-duplicated positive cultures from clinically significant sources were reviewed for time to positivity. Even though time required for recovery of different species varied, detection of growth was less than 14 days in 99.9% of cultures. Organisms recovered after 14 days had no clinical significance. ITS PCR yielded clinically significant positive results for culture negative samples from patients with high suspicion for fungal infection.

Manual of Clinical Microbiology recommends that cultures that screen for *Candida spp.* need to be incubated for no longer than 72 h, cultures that screen for dermatophytes need to be incubated for only 8 days, and cultures screening for all pathogens should be held for 4 weeks. With increased immunocompromised population and aggressive use of immunosuppressive therapy, fungal culture has to cover wide range of organisms for most specimen types. As a result, incubation of fungal culture for 4 weeks is a common practice in most of clinical microbiology laboratories. The extended incubation time not only delays reporting of negative culture result, but also increases the chance of recovering contaminant. Only seven samples grew fungal species after 14 day incubation. In all seven patients, the organisms recovered by culture were determined to be clinically irrelevant by treating physicians. Among the seven isolates recovered after 14 days, three of them were *Penicillium spp.* Although *Penicillium spp.* was the third most commonly isolated fungal species in our study, the clinical significance of the most cultures with *Penicillium spp.* was questionable. Our study showed that average 30% of culture positive samples had positive fungal smear. Smear positive samples with *Scedosporium spp.* and *Zygomycetes* accounted more than 44% of culture positive samples. However, only 1.2% of samples that grew *Penicillium spp.* were smear positive, which raise the question about clinical relevance of the species recovered during extended incubation.

A previous report examining the incubation time for recovering *H. capsulatum* in endemic area reported that up to 4-week incubation was required to recover the organism (8). Samples analyzed in the study included the samples from patients received anti-fungal treatments before samples collection. Delayed recovery of the dimorphic fungal species was not noted in our patients. In our study, dimorphic fungi species were recovered in 99 samples. The recovered isolates included 72 *Blastomyces dermatitidis*, 15 *Coccidioides immitis*, 9 *H. capsulatum*, and 2 *Sporotrichum schenckii*. Of these samples, 40.4% samples were found to be culture positive after 1 week, 22.2% were positive after 2 weeks, and 37.4% were positive after 3 weeks. All the samples were collected before treatment initiation. Our results indicated that fungal treatment can significantly delay the development of positive fungal culture.

We showed that clinical significant pathogens were detected in culture negative samples by ITS PCR in samples from 16 patients. Low sensitivity of culture has been reported for several important fungal pathogens. Reported rate of pathogen recovery from culture ranged from 8–31% (13). Culture-independent laboratory tests based on detection of antibodies, antigens, or metabolites have been developed, their application for diagnosis of invasive fungal infections suffers from lack of specificity, sensitivity, or being unable to provide species level information. ITS PCR followed by sequencing has become the culture-independent method of choice for the advantage of allowing for rapid detection and identification of fungi in clinical specimens. A recent study to evaluate the Performance of Targeted Fungal Sequencing for Culture-Independent Diagnosis of Invasive Fungal Disease reported the method had 62.9% of diagnostic yield in patients with suspected invasive fungal infections. In our study, the ITS PCR positive rate was much lower, only 0.2%. Two main factors may contribute the low positive rate. First, we included all samples that had been tested with ITS PCR regardless of the clinical suspicion for fungal infections. Because of the reported high sensitivity of ITS PCR for detection of fungi in clinical samples, the test was often used by clinicians as a rule-out test. The number of patients underwent testing who had true clinical suspicion for fungal infections was unknown. Second, we did not have minimal requirement for sample size. As ITS PCR was used as the last resort in clinical practice, the sample remains for PCR was scant.

Conclusion

Different from the studies that examined the fungal culture time and published before 2013, our study reviewed the data collected in the recent years, reflecting the laboratory practice in response to the increased incidence of invasive fungal disease as a result of the expansion of immunocompromised populations. In addition, the utility of ITS PCR as a last resort to assist the diagnosis of fungal infections was reviewed. Our study concluded that extending culture incubation beyond two weeks did not generate clinical relevant results. When culture failed to make laboratory diagnosis, ITS PCR produced clinical significant results.

Declarations

Authors' Contributions

AZ: conceptualization, methodology, investigation, formal analysis, data curation, and the original draft preparation

CQ: resources, manuscript review and editing

Conflicts of interest

The authors declare that they have no competing interests.

Funding information

None

Ethical approval

The human study protocol was approved by the institution review board of Northwestern University.

Consent for publication

Consent was obtained from participants for publication.

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