The Fluorescence in Situ Hybridization Technique (FISH) for Early Diagnosis of Fungal Sepsis in Immunocompromised Patients

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Abstract

Background: Sepsis is a serious clinical condition that characterized by a systemic inflammatory response syndrome. The mortality rate was reported to range between 13-20 million cases per year worldwide. In order to treat sepsis the detection of the causative pathogen is extremely crucial; especially fungal invasion in immunocompromised patients. Fungi are increasingly recognized as major pathogens in critically ill patients. Candida species and Cryptococcus species are the yeasts most frequently isolated in clinical practice.

Methods: Forty nine patients in ICU represented the material for this study and Fluorescence in Situ Hybridization (FISH) was evaluated as a method for diagnosis compared to conventional diagnosis methods. Important questions considered to be answered; these include sensitivity, accuracy, robustness, frequency of testing, experience needed, duration to get the results, promptness, and consequently the cost.

Results: FISH had sensitivity and specificity of 100% that compared to 100% specificity of traditional method and sensitivity of 92%. The mean times to diagnosis offer the detection of microbial growth in the automated blood culture system were 5 hours, and 6 days for detection by FISH and traditional microbiological approaches respectively.

Conclusion: The study revealed that FISH is a rapid and reliable method for direct identification and differentiation of fungal growth in blood cultures. FISH is relatively cheap, valid, and appropriate for daily routine work. FISH can elucidate that earlier fungal identification results in earlier appropriate antimicrobial therapy and in a better clinical outcome for the patients.

Key Words: Fluorescence in situ hybridization – FISH – Fungal sepsis – Immunocompromised.

Introduction

The sepsis syndrome is one of the leading causes of death in hospitalized patients. The mortality rate of septicemic patients varies between 30% and 70% and depends on several factors, including pathogen and host factors [1].

Invasive fungal infection is a major cause of morbidity and mortality in immunocompromised patients. Population-based surveillance estimates their prevalence at 12-17 per 100,000 populations [2]. Invasive candida infections are most commonly seen in critically ill patients in Intensive Care Units (ICU) and very low birth weight infants [3]. Reported mortality in patients with candidaemia ranges from 36 to 63%, although mortality in ICU patients has decreased in recent years; possibly due to more prompt initiation of antifungal therapy [4].

Fungi are one of four major groups of microorganisms (Bacteria, viruses, parasites, fungi). Most fungal infections occur because a person is exposed to a source of fungi such as spores on surfaces, in the air, soil, or in bird droppings. Deficiency in the body’s immune system defenses provides the right environment for fungal growth and invasion. Those immunocompromised host include organ transplant recipients, people who have HIV/AIDS, those who are on chemotherapy or immune suppressants, and those who have underlying condition such as diabetes or lung disease [5,6]. Invasive fungal infections are major causes of morbidity and mortality in critically ill patients. Foremost, among these is invasive candidiasis. In recent years, Invasive Aspergillosis (IA) and zygomycosis have emerged as major problems in susceptible critically ill patients. Risk factors for invasive fungal infections, included also disturbed anatomic barriers, suppressed antifungal host responses, and exposure to potentially opportunistic fungi [7].

Molecular techniques, particularly PCR-based approaches have been developed to detect fungi in a short period of time; these approaches include nested PCR, multiplex PCR, real-time PCR and microarray techniques. Although they have shown convincing results, the assays remain expensive,
and definitive results are guaranteed only after several hours by highly experienced microbiologists [8,9]. Fluorescence in Situ Hybridization (FISH) has already been successfully implemented in clinical microbiology for the identification of various pathogens, including fungi [10]. The hybridization of fixed fungi with fluorescently labelled oligonucleotide probes that are complementary to unique target sites or the ribosomal RNA allows direct microscopic visualization without prior amplification steps, even from blood culture smears [11].

**Aim of the work:**

This study is to demonstrate the application of FISH for the routine identification of primary causative agents of invasive fungal infections. The aim of the study was to evaluate FISH for the identification of fungi directly from positive blood cultures of patients with suspected fungal sepsis.

**Material and Methods**

This study was carried out in a major internationally accredited hospital in the eastern region in Saudi Arabia, Al-Mouwasat Hospital. The laboratory services are internationally accredited by the clinical pathology American Association. The intensive care units were the source of the patients who were included in this study.

Patients had risk factors that predispose for invasive or systemic fungal infections. These risk factors included the following:

- Mostly old age.
- Prolonged hospitalization.
- Mechanical ventilation for longer than three days.
- Broad spectrum antibiotics.
- Some with recent chemotherapy or radiation therapy for hematologic malignancy and solid neoplasms.
- Patients on parenteral hyperalimentation with central venous lines, central intravascular access devices and Foley’s Catheters.
- Patients with gastrointestinal tract surgery.
- Severe trauma.
- Burn.
- Hemodialysis; acute and chronic renal failure.
- Bacterial infections and broad-spectrum antibiotics.

Samples were collected from 49 patients who had referred to the microbiology laboratory between February 2014 and January 2015. Anonymized patient information was obtained from the computer system in the automated medical record of the hospital. This included, sex, age, place of residence, clinical specimen investigated, hematologic and chemistry blood tests results, HIV serology and Hepatitis B and C virus serology. These 49 cases had 49 blood cultures that had presented positive results of (CO2 production) in a blood culture system BACTEC 9120 (Becton-Dickson, Sparks, MD, USA); however these blood samples were negative for bacteria according to Gram stain analysis.

The detection and identification of pathogenic fungi were performed using traditional biochemical and micromorphological identification methods.

Blood cultures (without any previous treatment) were the source of prepared slides for direct microscopic examination.

Lactophonal cotton blue and 10% potassium hydroxide (KOH) were used for the visualization of general fungi cells. India ink was used for the visualization of Cryptococcus spp. capsules.

Cultures from 100-200ul samples of blood culture were grown using Sabouraud agar (BD Difco, Sparks USA) and Mycosel agar (BD Difco, Sparks USA) the fungal cultures were inoculated on Niger seed agar and CHROM agar Candida selective media (BD Difco, Sparks, USA). When the selective media were insufficient for the determination of the pathogens species, the isolates were subsequently subjected to micro-morphological and physiological tests using the fungal identification Kit AP120 (bioMerieux Vitek, Inc., Hazelwood, MO, USA).

**Detection and identification of pathogenic Fungi by FISH:**

The initial FISH reaction was performed using the panfungal probe, and then specific probes for each fungal species were used when fungal structures were found. These probes were chosen because of their high specificity and because of the absence of cross-reactions with other fungal species. The details of each probe used in the present study are presented in (Table 1). In addition to the probes the samples were counterstained with DAPT [4, 6-diamidino-2-phenylindole-dihydrochloride].

For the investigation of pre-incubated blood cultures, 0.5ul acetic acid (100%) was added to 5ml blood culture medium to lyse the erythrocytes. The suspension was centrifuged at 10,000 Xg for 5min. Then, the supernatant was discarded, and
Mohamed T. El-Kholy, et al.  147

the pellet containing cells was washed with 500μl of phosphate-Buffered saline (PBS; 130mM sodium chloride and 10mM sodium phosphate Buffer-PH 7.2-) and fixed for 4h with 4% w/v paraformaldehyde in PBS at 4 degrees Celsius after fixation, the cells were washed twice with PBS, suspended in one volume of PBS and one volume of cold absolute ethanol and stored at –20 degrees Celsius until use.

The FISH assay was performed as described by Amann [12,13]. The whole fixed cells were smeared onto precleaned microscopic slides and dried at 37 degrees Celsius for 20 minutes. Next, the slides were covered with hybridization buffer (0.9 M NaCl, 0.01% w/v SDS, 20mM Tris-HCl (pH 7.2), formamide and 1μM probe) and incubated at 46 degrees Celsius for 2h. After this period, the slides were washed with wash buffer (20mM Tris-HCl (PH 8.0), 0.01% W/V SDS, 5mM EDTA and NaCL) for 30min at 46 degrees Celsius. The concentrations of formamide and NaCl in the hybridization buffer and wash buffer, respectively, varied according to the probe (Table 1). Then, the slides were dried at 37 degrees Celsius for 20min, mounted in Vectashield solution (Vector, Burlingame, CA, USA) and examined using a Zeiss Axioskop 40 microscope (Zeiss, Jena, Germany).

Table (1): Information regarding the probes used in the present study.

<table>
<thead>
<tr>
<th>Target microorganisms</th>
<th>Probes</th>
<th>Sequences (5'-3')</th>
<th>Formamide content in hybridisation buffer (% v/v)</th>
<th>NaCl content in wash buffer (M)</th>
<th>Authors</th>
</tr>
</thead>
<tbody>
<tr>
<td>C.albicans</td>
<td>Caa1*</td>
<td>GCCAAGGCTTATACTCGCT</td>
<td>30</td>
<td>0.112</td>
<td>Kempf</td>
</tr>
<tr>
<td>C.glabrata</td>
<td>Cag1*</td>
<td>CCG CCA AGC CAC AAG GAC T</td>
<td>30</td>
<td>0.112</td>
<td>Kempf</td>
</tr>
<tr>
<td>C.parapsilosis</td>
<td>Capa*</td>
<td>CCTGGTTCGCCAAAAAGGC</td>
<td>20</td>
<td>0.225</td>
<td>Kempf</td>
</tr>
<tr>
<td>Aspergillus spp.</td>
<td>Asp*</td>
<td>TGATACATTCGAG</td>
<td>25</td>
<td>0.159</td>
<td>Wang</td>
</tr>
<tr>
<td>C. neoformans and C. gattii</td>
<td>Cne205*</td>
<td>CCAGCCCTTATCCACCGA</td>
<td>20</td>
<td>0.225</td>
<td>Martins</td>
</tr>
<tr>
<td>H. capsulatum</td>
<td>Hca1 *</td>
<td>AGTCGGAGGCTTTCAGCATGT</td>
<td>30</td>
<td>1.112</td>
<td>Silva Jr.</td>
</tr>
<tr>
<td>Fungi</td>
<td>Pan Fungal**</td>
<td>CTCTGGCTTACCCCTATT</td>
<td>30</td>
<td>0.112</td>
<td>Amann</td>
</tr>
</tbody>
</table>

Results

Forty-nine patients were collected in an ICU setup. Those 49 critically ill patients had invasive fungal infections with risk factors of disturbed anatomic barriers, suppressed antifungal host responses, and exposure to potentially opportunistic fungi. These forty-nine patients age was ranging from 23-94 years. The length of ICU stay was ranging between 7-54 days. Male to female was 32 to 17 cases samples were examined by conventional microscopy (Indian ink) and FISH were performed to confirm or exclude the presence of fungal agents. Nine samples tested positive by FISH but negative by conventional microscopy. Forty samples showed positive results with traditional microbiological approaches and FISH analysis. The identification of pathogens included the C neoformans complex (n=27), Histoplasma capsulatum (n=9) and Candida albicans (n=13). Traditional microbiological approaches and FISH analysis showed identical results for the blood culture materials in forty cases. However, the mean times to diagnosis offer the detection of microbial growth in the automated blood culture system were 5 hours, and 6 days for detection by FISH and traditional microbiological approaches respectively. FISH had sensitivity and specificity of 100% that compared to 100% specificity of traditional method and sensitivity of 92%.

Discussion

The present study demonstrated the application of FISH for the routine detection of the primary causative agents of invasive fungal infections in patients with critical illness in ICUs. These results agree with multiple worldwide, wide scale studies that have been implemented in clinical microbiology for the identification of pathogenic species of Candida and Cryptococcus [14-16].

Septicemia is a life threatening event which requires rapid appropriate therapy. The outcome for the patients with septicemia depends on factors including the septicemia-causing pathogen, and rapid microbiological diagnosis is desirable. In fact it is well established that a severe infection may proceed to a systemic inflammatory response syndrome that may continue in septic shock. While in the late phase of these events, immunomodulatory therapy, including anti-inflammatory cytokines
or cytokine antagonists as well as coagulation inhibitors and antimicrobials are essential for therapy; appropriate antimicrobial therapy is decisive in early phase. Clearly, rapid identification of the sepsis-causing pathogen is prerequisite for early appropriate antimicrobial treatment [17,18]. These facts explain the real need to have (FISH) to reach an early diagnosis. The sensitivity of (FISH) for fungal infections proved to be better than conventional microscopy (Indian ink) under their experimental condition. Also FISH most likely allowed the detection of fungi cells that had an altered capsular appearance caused by antifungal treatment [19,20]. A multi-centric approach to obtain the numbers of respective samples needed to perform a statistical comparison is necessary. Traditional microbiological assays require the sub-culturing of blood culture samples; microscopic assays including India Ink, lactophenol cotton blue and gram staining; and biochemical assays, including sugar assimilation, sugar fermentation and enzyme production tests. These assays are slow, requiring 3-10 days and trained microbiologists.

PCR-RFLP requires DNA extraction, PCR and electrophoresis to assess the PCR products. In addition, the PCR products must be digested by restriction enzymes, and the RFLP profiles must be evaluated. These steps require at least 20 hours and trained staff with experience in molecular techniques. FISH is robust, easier to perform, and considerably faster, with a time-to-results up to 5 hours [21,22].

In this study we used FISH combinations of six adopted probes which give a great advantage for the diagnosis of histoplasmosis. Histoplasma capsulatum cultures should be manipulated under high biosafety containment conditions. FISH demonstrated the capability of identifying the fungi directly in biological samples and did not require sub-cultures; these characteristics are essential for conventional diagnosis [23]. In this study nine blood cultures presented "microbial growth" in the blood culture system but did not present fungal growth (Traditional microbiological assays). The false detection of microbial growth by blood culture systems has been previously described [19]. The simultaneous lack of detection by FISH demonstrated the adequate specificity of the FISH technique. The combination of probes in this study represented a limitation that could not cover other pathogens; however, this limitation was mitigated because the choices of probes used was based on research by Souza et al., [24], who demonstrated the predominated species of fungi that cause invasive mycoses.

Early diagnosis of invasive fungal infections and prompt initiation of antifungal therapy are essential to reduce morbidity and mortality. Multiple retrospective studies have examined the crude mortality in patients with Candidaemia and identified rates ranging from 46% to 75%. In many instances, this is partly caused by severe underlying comorbidities. Resource use associated with this infection is also significant. Estimates from numerous studies suggest the added hospital cost is as much as $40,000 per case. Overall attributable costs are difficult to calculate with precision but have been estimated to be close to one billion dollars in the United States annually [25-27]. Studies have demonstrated that FISH is a useful tool for environmental microbiology studies. The improvement of this technology motivated the development of several works in clinical microbiology. The detection of Candida spp. motivated most of these studies and resulted in the production of commercial kits. FISH probes were developed for detecting Cryptococcus Species and H. Capsulatum. Additionally these studies have clearly demonstrated the FISH technique is effective with biological samples that present high fungal cell content [20, 25,28].

Conclusion:

Significant advances in the early diagnosis of invasive fungal infection by using noninvasive techniques including FISH have the potential to impact on empirical strategies when incorporated into care pathways. These techniques may be used to guide pre-emptive therapy and reduce unnecessary empirical antifungal use. Benefits from this approach include not only reduced drug acquisition costs but also reduced morbidity and mortality from drug-related adverse events and decreased hospital length of stay. The ultimate goal will be a reduction in fungal-related death and improved overall survival. Optimal patient management will require identification of the microorganism causing invasive fungal infection specifically in those high risk groups. Identification of fungal agents i.e. Candida spp. Cryptococcus spp, and H. capsulatum using the routine procedures was very important to assess and manage critical ill patients in ICU. FISH was proved to be a valuable, robust, fast and easy to perform tool that can be readily implemented in the diagnostic routine of hospital laboratories.

References


150 FISH Technique for Early Diagnosis of Fungal Sepsis in Immunocompromised Patients